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J. A. CALLOW
School of Biosciences,
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An Epidemiological Framework for Disease Management

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ABSTRACT

There are two broad approaches to the management of disease. One is largely empirical, driven by experience and practice. The other, which is the focus of this review, is designed around an epidemiological framework in which the mechanisms responsible for an epidemic are identified and used to target, improve and deploy methods to reduce the risk of disease. This, in turn, demands an understanding of the processes that control the invasion and persistence of inoculum and disease. The review is driven from recent experience on soil- and air-borne disease, including disease transmitted by vectors, as well as insights gained from animal and human disease. It addresses the management of disease within single fields as well as the regional deployment of control. Hence we seek to answer questions such as: how much inoculum is required to initiate a focus, what is the minimum amount of infection needed to initiate an epidemic, through to, how can a new disease invade, will it persist and, if not, what is the time to extinction? Success depends on balancing biological detail with epidemiological insight in order to capture the essential dynamics of epidemics. We begin with a simple non-linear model for the transmission of infection that incorporates primary and secondary infection, together with changes in host susceptibility. By fitting the model to data for biological control of a damping-off disease, we show how to identify which epidemiological mechanisms are targeted by the control agent and how these might be enhanced. Variability in the success of biological control is captured in a stochastic version of the model, which allows prediction not only of the average level of disease but also the risk of failure. The model has broad applicability to a range of diseases and types of control. Host growth is shown to be important not only in controlling the supply of susceptible tissue but more subtlely in speeding up or slowing down epidemics as plants respond to disease. Methods are proposed to link inter- and intra-seasonal dynamics in the presence of sudden disturbances characteristic of sowing and harvesting of crops that are an integral part of agricultural production but frequently ignored in epidemiological analyses. Methods are described to extract epidemiologically important information from complicated models. Simplification again leads to criteria for the risks of invasion and persistence of inoculum and disease. The invasion of fungicide-resistant isolates is discussed and methods described to predict extinction times for resistant strains. The spatial structure of susceptible crops is then considered, with attention focused on metapopulations to describe the spread of disease in the landscape. Finally we discuss criteria for invasion and disease management in dynamical landscapes, when susceptible crops are rotated and the connectivity between contiguous fields of susceptible crops changes. The connectivity between susceptible crops leads to a discussion of percolation for which we show that there is a critical probability for transmission between neighbouring fields for disease to spread.

I. INTRODUCTION

Epidemics are highly non-linear. They involve a hierarchy of scales. They are inherently stochastic and they occur in heterogeneous environments. Few growers, however, would seek the advice of a mathematician in deciding how to manage disease! Empirical field trials, together with surveys of farm practice, still largely govern the way that we manage disease. These strategies are often successful, but the chances for mistakes are considerable and success is often transient. Unexpected outbreaks of disease still occur: control is incomplete, short lived, uneven and patchy. The parasite is also subject to large selection pressures. Genetical resistance frequently breaks down. Resistance to fungicides

is a recurrent and serious problem, while biological control remains beguilingly unsuccessful.

Underlying many of these problems are the concepts of invasion and persistence. We still do not understand why some diseases take off and others do not, or why some races persist while others disappear. Yet this is fundamental to the management of disease. Invasion starts at the microscale around a single plant with the arrival of inoculum. It extends to the expansion of a disease patch, to multiple patches in a field and on up to the regional spread of disease. Most crop management is aimed at controlling disease in a single field, or equivalently a glasshouse, plantation or orchard, but this requires some understanding of the smaller-scale processes that lead to epidemic development. *How much inoculum is required to initiate a focus? How fast can a focus expand? Why do foci appear and disappear? What is the minimum amount of infection needed to initiate an epidemic or to sustain crop loss?* Management of disease also encompasses regional deployment of control, in order to minimise the risk of spread of pesticide and varietal resistance or the spread of a new disease (Fry *et al*., 1992; Zhu *et al*., 2000). Important questions are: *Can a new race invade? How long will it take to invade? Will it persist? Will it coexist with the wild type? What is the time to extinction?*

To answer many of the questions posed above, it is necessary to identify the spatial and temporal dynamics of disease that underlie invasion and persistence. This is far from trivial because it encompasses not only the dynamics of the pathogen, but also those of the host and frequently too of vectors or of specific microbial antagonists, all interacting in a variable environment. How can we construct a theoretical framework? Using recent experience drawn from our work on soil- and air-borne disease, including disease transmitted by vectors as well as animal and human disease, I present some suggestions. In doing so, I wish to make four points:

1. simple non-linear models can be used to capture the temporal dynamics of epidemics in a way that enables us to identify criteria for invasion;
2. variability plays a key role in both invasion and persistence of disease and can be represented in relatively simple stochastic models;
3. host growth also affects invasion and persistence and can easily be incorporated into epidemic models;
4. the models must be elaborated to include spatial variation in order to predict the regional risk of disease.

The literature cited is selective. It is designed to support the principal ideas and to introduce the reader to literature in related disciplines.

II. CAPTURING THE ESSENTIAL DYNAMICS OF EPIDEMICS

Capturing the essential dynamics of epidemics involves making difficult decisions about what to include and what to leave out. Decisions encompass how

much biological detail to include in the model, what dynamical issues have to be addressed and how the model will be used. Here I focus on invasion and persistence and on variability within and between epidemics. Some of the principal biological and dynamical considerations, together with applications, are summarised in Table I.

Decisions about the biological and dynamical issues and the use to which the model will be put lead naturally to model structure. Much has been written about modelling strategy in epidemiology and ecology. It is not wise to be overly prescriptive about model structure: needs and fashions change. So does technology, especially the power of computers – opening up new possibilities of analyses. Nevertheless, the arguments for parsimony, keeping the numbers of parameters and variables in the model as few as possible, are compelling because it gives some chance of identifying which demographical factors are important in controlling the epidemic. Early excursions into multivariate and multiparameter models, with the advent of fast computers towards the 1970s, were exciting and offered much promise (Waggoner and Horsfall, 1969; Waggoner *et al.*, 1972). These multivariate, multiparameter models, however, have proved rather intractable for practical and strategic use in controlling disease. Current preoccu-

TABLE I

Summary of biological and dynamical considerations for construction of epidemiological models, together with selected applications

BIOLOGICAL ISSUES: WHAT SHOULD BE INCLUDED IN THE MODEL?

Dynamics of inoculum,
Dynamics of primary infection from resident inoculum or immigration,
Dynamics of secondary infection between infecteds and susceptibles,
Host dynamics including the rate of production of susceptible tissue,
Host responses to infection, including the effects of disease on the supply of susceptible tissue,
'Quenching' due to change in host susceptibility or onset of unfavourable conditions,
Vector or antagonist dynamics,
Genetical structure of the parasite population,
Definition of scale for the susceptible and infected units, ranging from lesions, leaves, roots, stems, single plants to whole fields.

DYNAMICAL ISSUES

Non-linearity	(*small changes that have big effects on epidemics*)
Stochasticity	(*chance variation within and between epidemics)*
Spatial heterogeneity	(*local and regional changes that affect spread*)
Temporal heterogeneity	(*daily and seasonal changes that affect spread)*
More than one scale	(*from micro- through field to regional scale)*

APPLICATIONS OF THE MODELS: WHAT WILL THE MODEL BE USED FOR?

To predict invasion of a new pathogen or race.
To predict the consequences of control strategies on:
– invasion and persistence;
– crop loss.
To predict the *risk* of disease.

pations now centre upon whether or not models are: deterministic or stochastic, non-spatial or spatial, as well as the degree of biological complexity of the models. The latter varies with objective and scale. In tracing the continental spread of a parasite, it may be sufficient to treat fields of susceptible crops as susceptible or diseased. Finer detail about the level of infection within individual fields may be superfluous (van den Bosch *et al.*, 1999; Zadoks, 1999) when we simply need to know whether or not a field is capable of infecting its neighbours and for how long the risk continues. To predict the effectiveness of a biocontrol agent in a horticultural crop, however, it may be necessary to include more detail about spatial pattern and amplification of the hyperparasite, for example to consider what happens at the level of the individual plant (Bailey and Gilligan, 1997) as well as in a population of plants. The question of scale has so far received relatively little formal treatment in botanical epidemiology (Onstad, 1992; Onstad and Kornkven, 1992; Thrall and Burdon, 1999; Zadoks, 1999), although it sits centrally in ecological theory (Newman and Watts, 1999; Levin, 2000; Mack *et al.*, 2000). It will undoubtedly become more important as we consider modern approaches to epidemiology.

A. A SIMPLE MODEL

Conventionally, epidemiology has concentrated on modelling diseased (or infected) tissue leading to simple deterministic models for monotonic growth, such as the monomolecular, logistic or Gompertz models (Campbell and Madden, 1990; Kranz, 1990; Hau *et al.*, 1993). Many of the models can be solved analytically and have provided very useful methods for comparing the effects of treatments on the dynamics of disease (Gilligan, 1990a,b). Now, however, most models are derived from within a generic framework for the flow of tissue through different states from susceptibles to infecteds and ‘removals’. Some examples are shown in Fig. 1. The formulation is not new: variants of the *SIR* format were used skilfully and elegantly in medical epidemiology at the beginning of the last century (Kermack and McKendrick, 1927) to predict thresholds for disease and were introduced in botanical epidemiology by several key workers (Vanderplank, 1963; Zadoks, 1971; Jeger, 1982), but they have only more recently become widely established (Campbell and Madden, 1990). They can be elaborated to allow for extraneous sources of infection, notably primary infection from a reservoir of inoculum in soil or immigration in aerial disease (Fig. 1). Although this approach appears to distinguish them from human and animal disease, it turns out that botanical epidemiology can introduce useful concepts into the broader area of epidemiology. One important feature in using the models is the selection of an appropriate and measurable host unit (Table I). This may be an entire plant, but more frequently it will be a leaf, stem or root, or perhaps an arbitrarily defined area of tissue. Where there is significant lesion growth, the form of the model can be adjusted to take this into account. A mathematical treatment of this is given in Gilligan and Kleczkowski (1997).

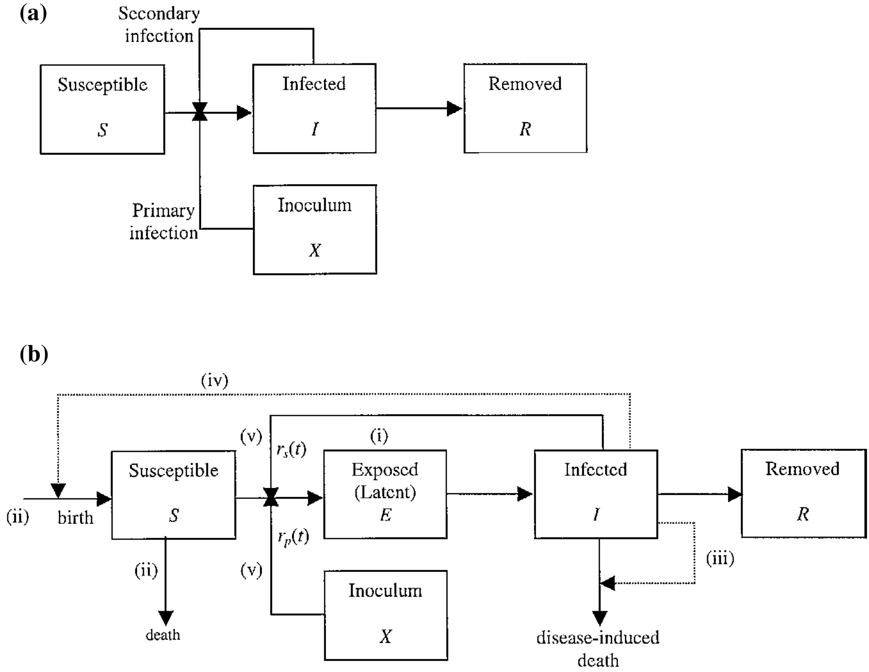


Fig. 1. Schematic representations of an *SIRX* model for the flow of susceptibles (*S*) to infecteds (*I*) and removals (*R*), with external inoculum (*X*). An individual defines the unit of population: it may be a plant but more frequently it will be a stem, leaf or root. (a) Simple scheme showing two sources of infection: primary infection through contact between susceptibles with external inoculum and secondary infection represented by contact between infected and susceptible individuals. (b) Some elaborations of the scheme are shown with (i) an additional compartment for latent (*E*, exposed) infection, (ii) vital dynamics (birth and death) of the susceptible host population, (iii) pathogen-induced death of infected hosts, (iv) disease-induced host dynamics, and (v) quenching of the infection rates.

This is not the place for an exhaustive treatment of models, instead I select below some examples for illustration. We begin with a model for primary and secondary infection with 'quenching' due to change in susceptibility of the host. The model is given by:

$$\frac{dI}{dt} = (r_p X + r_s I)s(t)(N - I), \quad (1)$$

in which r_p and r_s are the rates of primary and secondary infection respectively, X is the density of initial inoculum, N is the total number of hosts, I is the number of infected hosts and $s(t)$ is a measure of the change in susceptibility of the host. Kleczkowski *et al.* (1996) first used a model of this form to characterise the effects of the inclusion of a biocontrol agent, *Trichoderma viride*, on the

spread of damping-off disease of radish caused by *Rhizoctonia solani*. Two important features emerge from the analysis:

1. 'quenching' in the form of the characteristic change in susceptibility of the host, here represented simply by $s(t) = s_0\exp(-at)$, can have a profound effect on the epidemic, limiting the asymptotic level of disease far below the notional carrying capacity of $I = N$;
2. small changes in the parameters or in the initial conditions can lead to markedly different levels of disease. This dynamically generated variability (Kleczkowski *et al.*, 1996) reflects the response of disease to small environmentally controlled variations, and is discussed below.

Restriction in the carrying capacity below the upper limit of the total number of plants or plant parts has, of course, long been recognised (Jeger, 1984, 1986) but it has frequently been ignored in the analysis of epidemics (see the treatment in Gilligan, 1990b), leading to erroneous conclusions about disease dynamics. This is simply shown when model fitting is used to infer the effect that a treatment such as a fungicide has on disease control. Naïvely assuming that the epidemic can achieve 100% infection for all treatments by ignoring changes in susceptibility of the host can lead to assumptions that control is influencing one of the rate parameters, when it is really influencing the rate of change of susceptibility of the host. Basing future control strategies on a misconception about which parameter is affected will lead to wasted effort in research and possibly failure in control. Careful thought about the biology can usually avoid this, although parameter estimation for epidemiological models is still in its infancy.

B. INTRODUCING VARIABILITY

The model for primary and secondary infection, as with most others, can be written in stochastic form (Gibson *et al.*, 1999) to take account of demographic stochasticity, that is in the chance differences between repeated challenges of susceptibles by inoculum or infecteds. Thus, the probability that a new infection occurs in a short time interval is given by:

$$\Pr[I(t+\mathrm{d}t) = I(t)+1] = (r_p X + r_s I(t))s(t)(N - I(t))\mathrm{d}t. \tag{2}$$

Although this equation is similar to eqn (1), it is easy to show that the deterministic and stochastic models are *not* the same. Using $\langle\ \rangle$ to denote expectation or averaging, we rewrite eqn (2) to compare it directly with eqn (1) as

$$\frac{\mathrm{d}\langle I\rangle}{\mathrm{d}t} = \langle (r_p X + r_s I)s(t)(N - I)\rangle. \tag{3}$$

By multiplying out the right-hand sides of eqns (1) and (3) it is easy to show that since $\langle I^2 \rangle \neq \langle I \rangle^2$ (i.e. the mean of the squares is not the same as the square of

the mean) the deterministic version is not merely the average of the stochastic version and the two models differ. The difference arises because of the intrinsic nonlinearity of the model (conferred by the terms in I^2). It leads to a richness in analysing epidemics but also to a source of confusion between the two forms. Following earlier work by Kleczkowski *et al.* (1996) for the deterministic model in which parameters were allowed to differ between epidemics, Gibson *et al.* (1999) used a demographic stochastic model to analyse the evolution of the probability distribution for replicate epidemics of damping-off. This is important for two reasons:

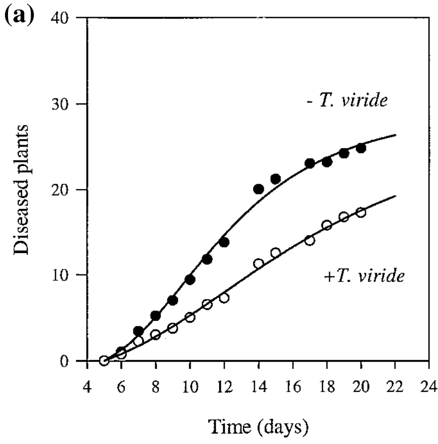
1. it allows us to model the variability between epidemics and so to predict the risk of disease under different conditions;
2. it allows prediction of the proportions of sites, which may be fields, that will have no disease as well as providing measures of the average levels of disease as they change over time.

The results are illustrated in Figs 2–4. Inspection of the average disease progress curves (Fig. 2(a)) shows that the biological control agent inhibits disease progress. Many studies would stop at this and assert a treatment effect usually with a simple analysis of variance or *t*-test for final levels of disease. Inspection of the individual disease progress curves for each replicate epidemic, however, provides much more insight into the dynamics of control (Fig. 2(b)). Clearly there is a large amount of variability among replicate epidemics. Sometimes the biocontrol agent controls disease very well, while at other times it does no better than in most of the uncontrolled epidemics. Conventionally, experimentation has been designed to minimise variability *within* treatments. This has been done for very good reasons, in seeking to detect variability *between* treatments. Unfortunately, this leads inexorably to a focus on average disease progress curves and so hides the intrinsic variability of the system, which is 'the very stuff' of epidemics.

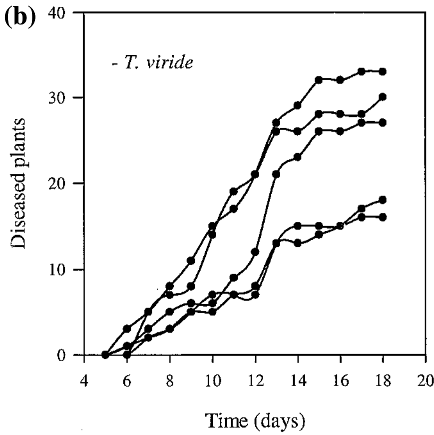
Variability is one of the most striking features of epidemics. No matter how carefully we control conditions, variability within and between epidemics persists. Variability occurs, of course, in the field and in field trials, but also in microcosms in the laboratory. Historically this variability has been attributed to environmental factors: temperature fluctuates, soil conditions differ from site to site, or varieties differ in susceptibility. Undoubtedly these are all true, but underneath lies a set of demographic rules by which pathogens and their hosts interact. These interactions are inherently stochastic (Shaw, 1994). They are governed by the probability of contact between inoculum and susceptible hosts, by variability in latency and in infectiousness. It is upon this demographic stochasticity that environmental variation interacts. I therefore argue that if we understand the population interactions then we can begin to understand variability and perhaps to improve the management of disease.

By fitting the stochastic model given in eqn (2) to data from experiments even with relatively low levels of replication, it is possible to predict what will

(a)



(b)



(c)

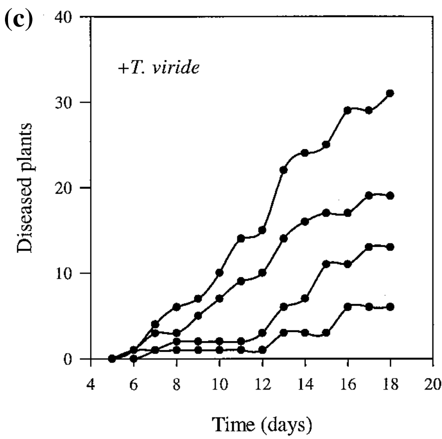


Fig. 2. (a) Data for the average disease progress curves for the colonisation of radish seedlings by *R. solani* show that the presence of the biological control agent, *Trichoderma viride*, can slow the epidemic, but the averaged data hide the variability between replicates, shown in (b) and (c).

happen in a large number of replicate epidemics and, hence, from this to predict the risk of disease (Gibson *et al*., 1999). Figure 3 shows the evolution of the probability distribution for damping-off of radish by *R. solani* in the presence or absence of *T. viride.* Now we see that *T. viride* is successful in deflecting the distributions downward towards zero, but that while there are likely to be startling successes in the biological control of disease, there will also be startling failures. This occurs because of the inherent variability of the system, which affects the chance of infection. Moreover, because epidemics are non-linear, small changes at a crucial stage in the epidemic can lead to large differences later on as disease progress curves diverge, even under highly controlled conditions.

Gibson *et al.* (1999) used maximum likelihood estimation to identify which epidemiological parameters were most affected by the addition of the biological control agent. There were three parameters, the rate of primary infection (r_p), the rate of secondary infection (r_s) and the rate of quenching (a), from which r_p emerged as the most likely candidate. (The parameter s_0 was subsumed into r_p and r_s since it was assumed to affect both equally.) It is important to note, however, that the identification of r_p as the critical parameter affected by *T. viride* depends on the model, and, of course, the data. The model fitted here (eqn (2)) is presented in demographic, stochastic form. That is, each new infection is a chance event, governed by the epidemiological parameters (r_p, r_s and a) which are assumed to be constant through time and across all replicates. In the earlier work (Kleczkowski *et al*., 1996), we analysed the model based on eqn (1) for a specific form of environmental stochasticity, in which we assumed that one or more of the parameters differed from replicate to replicate. This may occur because of small differences in growth conditions between the replicates. Under this assumption, Kleczkowski *et al.* (1996) showed that it was possible that s_0 may have been differentially affected by the biological control agent (Fig. 4). This implies that both r_p and r_s may have been affected. The models represent two contrasting systems for demographic and environmental stochasticity in which the parameters are assumed to be constant (Fig. 3) or differ (Fig. 4) between replicates. Inevitably, disease in the field will reflect both environmental and demographic stochasticity. Finding that more than one model fits the same data is not unusual, and neither does it impugn the modelling approach, providing it is rigorously done. What is of practical importance is the ability to predict variability among replicate epidemics over time. Each model allows the computation of this once the parameters are estimated from the data.

Disentangling the underlying mechanisms for how biological control occurs demands additional experimentation at the individual level. Support for this conclusion comes from independent experiments in which we monitored the probability of infection of individual plants in the presence and absence of *T. viride.* These experiments characterise the pathozone dynamics (Bailey and Gilligan, 1997; Gilligan, 1985) of infection and showed that *T. viride* could control the dynamics of primary infection (Fig. 5), whereas there was no effect on pathozone dynamics for secondary infection. Consistency in this form between

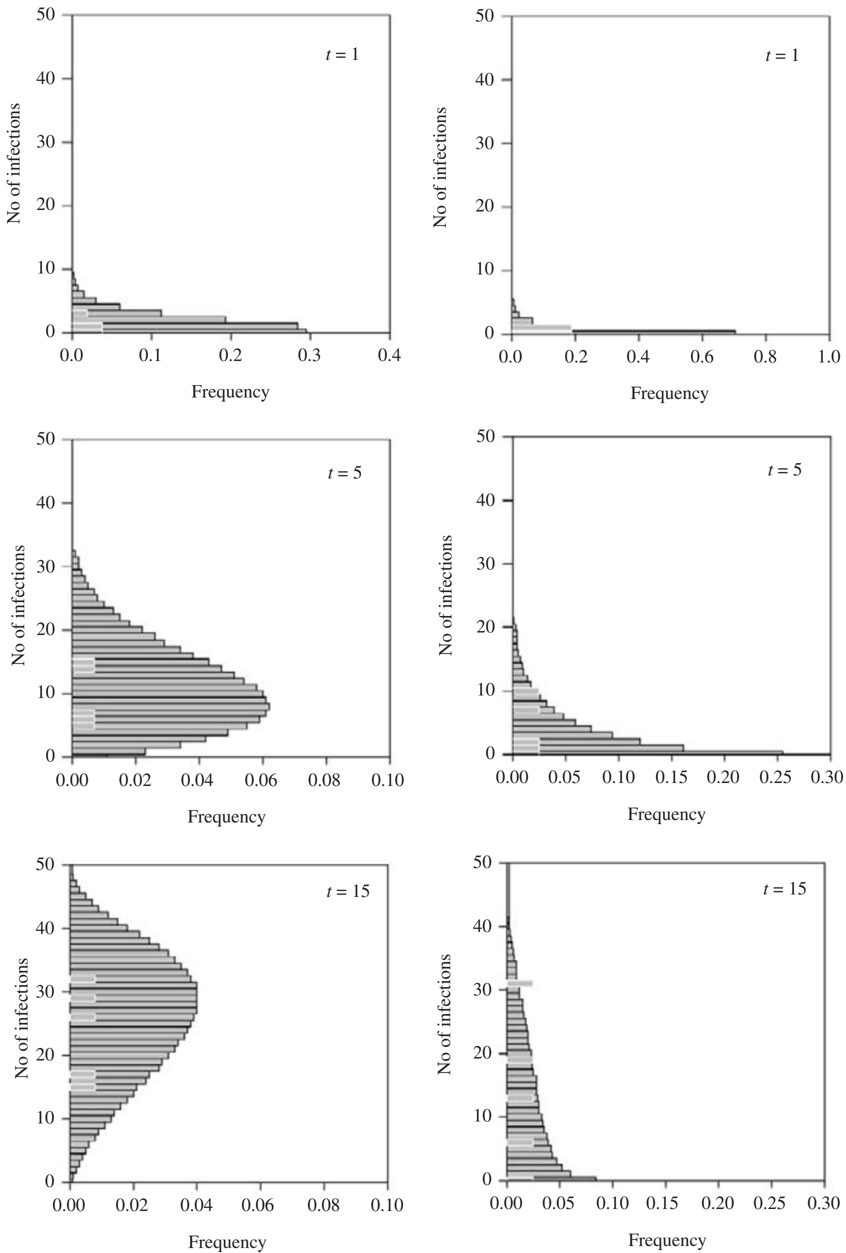


Fig. 3. A demographic stochastic model allows the estimation of the evolution of the probability distribution for the replicate epidemics of damping-off disease of radish by *Rhizoctonia solani* over time. Lightly shaded boxes represent experimental data. The presence of a biological control agent, *Trichoderma viride*, skews the distribution but still fails to control some epidemics. Data and analyses from Gibson *et al.* (1999).

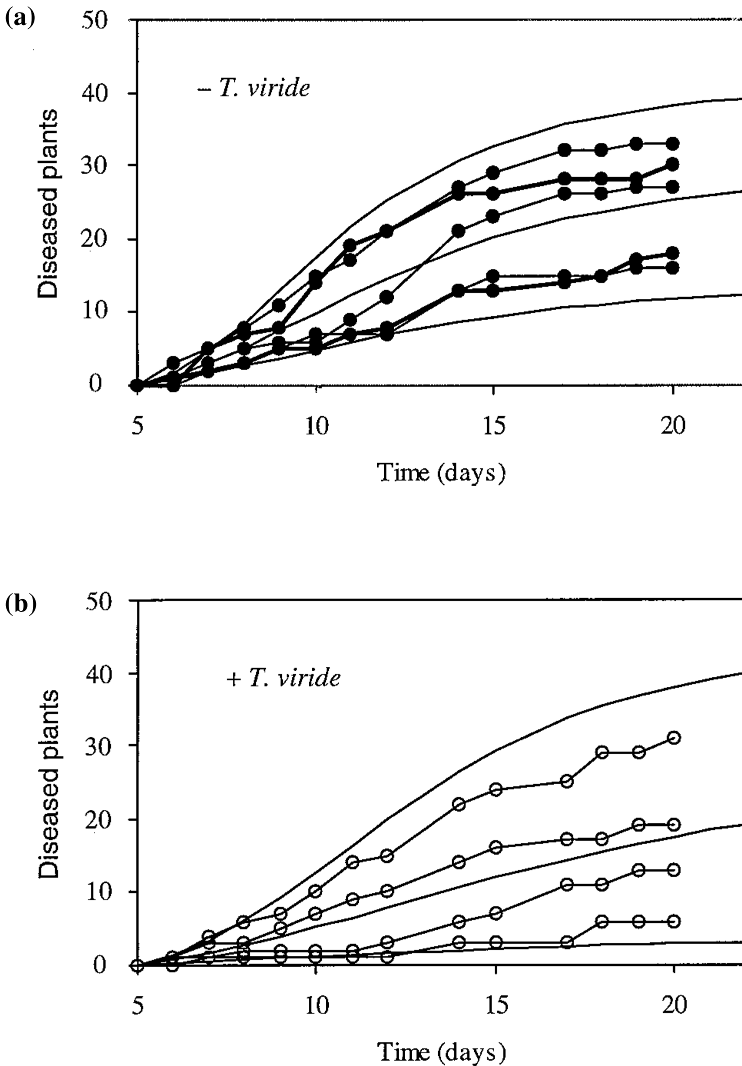


Fig. 4. The figure shows the result of fitting a deterministic model, but with allowance for small differences in parameter values between replicate epidemics, sufficient to give dynamically generated variability (Kleczkowski *et al.*, 1996). Smooth curves show median and 95% intervals predicted by the model under the assumption that s_0 varies by ± 31% in the absence of *T. viride* (a) and by ± 65% in the presence of *T. viride* (b). Reproduced with permission from Kleczkowski *et al.* (1996).

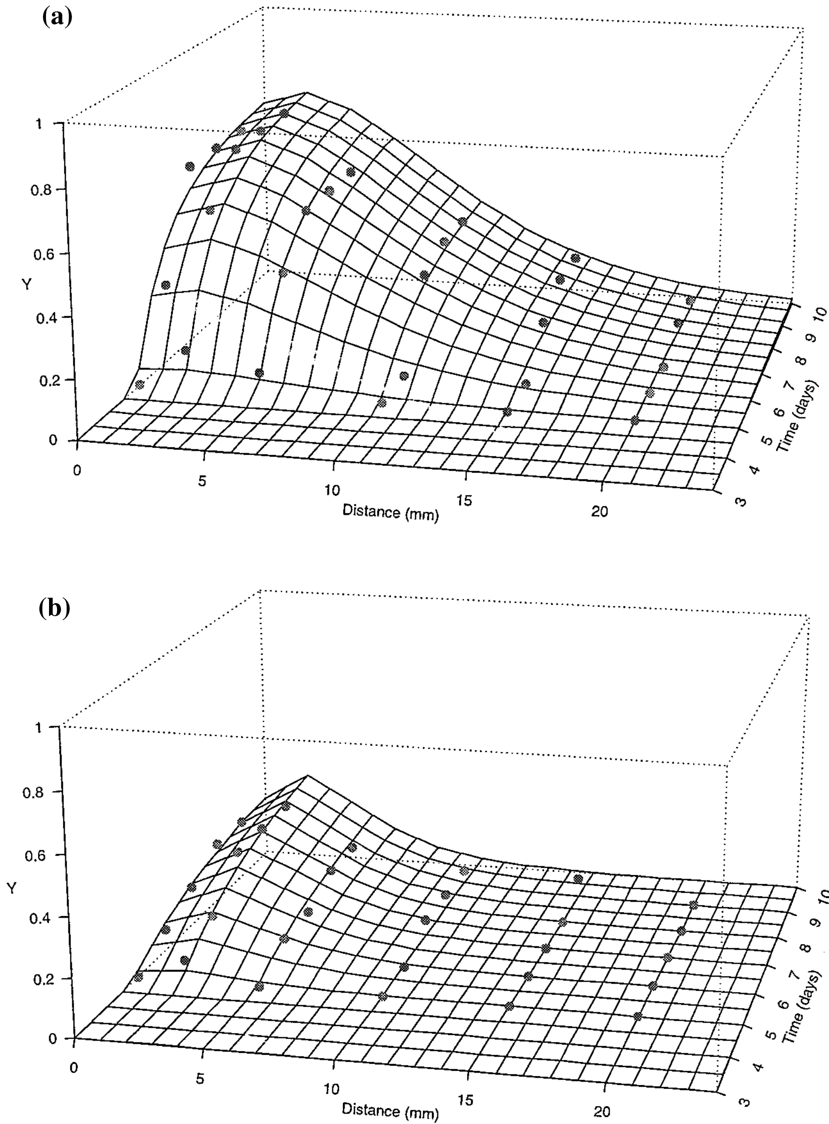


Fig. 5. Spatio-temporal dynamics of the pathozone for primary infection of radish hypocotyls by inoculum of *Rhizoctonia solani* in (a) the absence and (b) the presence of the biological control agent, *Trichoderma viride*. The dots represent experimental data and the surface is a theoretical approximation obtained by fitting a three-dimensional non-linear model to the data. The surface shows how the probability of infection changes over time and distance from the host. The dip close to the host arises because of disruption of the fungal hyphae as the seed germinates. Hyphae from more distant inoculum arrive after disruption is complete. Adapted, with permission, from Bailey and Gilligan (1997).

individual-based and population-based experiments therefore currently favours the analysis based on demographic stochasticity, but more work is under way to assess the evidence.

C. INTRODUCING MORE BIOLOGICAL COMPLEXITY

The simple models introduced above ignore the dynamics of host growth in which the availability of susceptible tissue changes throughout the season as new leaves and roots are produced, grow and senesce. These dynamics can be captured in two main ways. First is a simple host–parasite model that keeps track of the biomass, density or, less frequently, numbers of hosts (which may be whole plants, leaves, roots or other root organs) and measures of the parasite biomass (usually assessed as lesions, and less frequently as spores) (Gilligan and Kleczkowski, 1997; Gubbins and Gilligan, 1999). A second and more widely used framework is the compartmental, *SEIR,* format in which plant organs pass through notional compartments as they become susceptible (S), exposed but not yet infectious (E), infected and infectious (I) and removed (R) through death or exceptionally recovery (Gilligan, 1994). The elaboration of the models comes at the cost of additional parameters which have to be estimated. These include the latent and infectious periods, birth and death rates for the host and the transmission parameter, together with the parameters that capture the effect of disease on host growth. Of these the transmission parameters and the host–response parameters are the most difficult to estimate.

Here, for brevity, I neglect the latent and removed class and merely expand the model to S and I with free-living inoculum (X), following Gubbins *et al.* (2000),

$$\begin{aligned}\frac{\mathrm{d}S}{\mathrm{d}t} &= r\,(S,I,X) - Xf_p(S,X) - If_s(S,I),\\ \frac{\mathrm{d}I}{\mathrm{d}t} &= Xf_p(S,X) + If_s(S,I) - \mu I,\\ \frac{\mathrm{d}X}{\mathrm{d}t} &= gI - hX.\end{aligned} \tag{4}$$

This model incorporates terms for primary and secondary infection ($f_p(S,X)$ and $f_s(S,I)$) in the form of functional responses of the pathogen to changes in host density (Antonovics *et al.*, 1995). They can equally well be represented by forces of infection $\lambda(S,X)$ and $\lambda(S,I)$. The two forms are related for secondary infection by $If_s(S,I) \equiv \lambda(S,I)S$: preference for one or the other depends on mathematical and conceptual convenience (Gubbins *et al.*, 2000; Park *et al.*, 2001). Sometimes one form can lead to more intuitive understanding of underlying epidemiological mechanisms than the other. For example, Gubbins and Gilligan (1997b) have argued that the functional response leads to a more transparent analysis of invasion behaviour when there is a non-linear contact between host

and parasite (see section V.B.2). Host reproduction is represented in eqn (4) by the function, $r(S,I,X)$, which includes the effects, if any, of a host response to infection load (see section II.C.1, with further detail in Gilligan (1994) and Gilligan *et al.* (1997)). The parameter μ is the death rate for infected hosts and includes both natural and disease-induced mortality, g is the parasite reproduction rate and h is the parasite death rate.

1. Balancing Host Growth, Primary and Secondary Infection

A simpler version of the model in eqn (4) was used by Bailey and Gilligan (1999), together with analysis of field and microcosm experiments to identify crucial phases in the dynamics of take-all disease in wheat. Here the supply of susceptible host tissue is an important determinant in the dynamics of disease. The analyses show that there are two phases in the dynamics of epidemics of take-all (Fig. 6). There are:

1. an initial phase of primary infection by *Gaeumannomyces graminis* as seminal roots grow through the soil and encounter inoculum;
2. a second phase in which there is an accelerating rate of secondary infection stimulated by the increase in the availability of infected tissue, as a source of secondary inoculum, and the availability of more susceptible tissue for infection (Bailey and Gilligan, 1999).

The model for take-all is given by:

$$\frac{\mathrm{d}N}{\mathrm{d}t} = r_n f(N),$$

$$\frac{\mathrm{d}I}{\mathrm{d}t} = \left(r_p X + r_s I\right)S,$$

$$\frac{\mathrm{d}X}{\mathrm{d}t} = -r_d X,$$

where $N = S + I$. The model exhibits a cascade from the dynamics of inoculum which controls primary infection that in turn influences the cycles of secondary infection. Both types of infection depend on the rate of supply of susceptible tissue to infect (Fig. 6). Here we assume that inoculum for primary infection comprises a reservoir of colonised fragments of host tissue that is replenished upon harvest and cultivation at the end of the season. Thereafter, the reservoir decays monotonically during the ensuing growing season. This effectively quenches the rate of primary infection. Depending on the relative rate of secondary infection, this can lead to a temporary plateau in the dynamics of disease (Fig. 6) that reflects the balance of demography of wheat growth and infection and not, as many had thought previously, simply reflecting an environmentally-induced delay associated with lower temperatures in the winter. The plateau is therefore a demographic feature of the epidemic and not simply an environmentally forced freezing of dynamics. The plateau of primary infection is

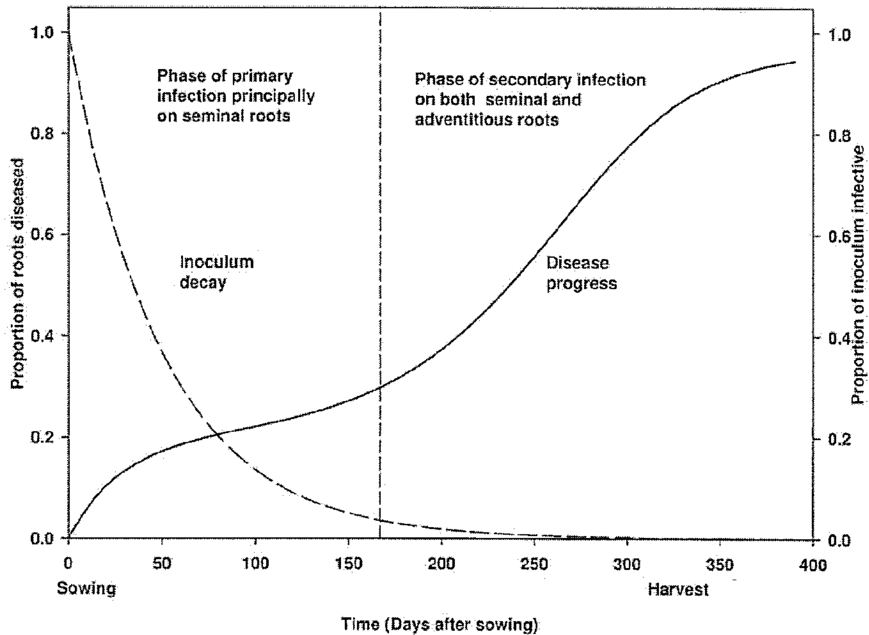


Fig. 6. Schematic representation of the epidemiological significance of successive phases of primary and secondary infection in the dynamics of take-all disease of wheat. The initial phase of primary infection arises from inoculum that survives on previously colonised root and stem material from the previous crop. This source of inoculum decays over time so that primary infection is restricted to the early phases of host growth. The amount of primary infection and the availability of susceptible adventitious roots each influence the subsequent phase of secondary infection, and whether or not primary infection 'takes off'.

the springboard from which secondary infection takes off as more adventitious roots are produced in the spring (Bailey and Gilligan, 1999). Now the plant has a chance to grow away from the infection if it can produce roots fast enough. Knowledge of the balance between these two phases is currently being used to optimise novel chemical and biological methods for disease control (Colbach *et al.*, 1997; Schoeny and Lucas, 1999). It follows that the amount of inoculum available for initiation of primary infection is an important determinant of infection that can cascade all the way up the epidemic to influence the final level of disease (Fig. 6).

2. Primary Infection and the Initiation of Soil-borne and Aerial Epidemics

For many soil-borne pathogens, within-season dynamics may be regarded as yielding an amplification of primary inoculum for infection of the next susceptible crop. That is, inoculum remains trapped within the decaying plant until it is released by cultivation. Many, indeed most, pathogens continually replenish the reservoir of inoculum during the epidemic as resting spores are released from

dying hosts. This can lengthen the phase of primary infection. It can also lead to the initiation of new foci as inoculum disperses to new fields. A critical balance between primary and secondary infection is important not only for soil-borne pathogens but also for many aerial pathogens in which there is a reservoir of inoculum that initiates primary infection. Here primary infection refers to immigration of inoculum from outside the system, for example from potato clamps for *Phytophthora infestans* or long-distance movement of spores of *Puccinia striiformis* (Hovmøller *et al*., 2002). Gibson (1997) has also shown how to discriminate primary from secondary infection using two snapshots of disease maps in the dynamics of Citrus tristeza virus. This external input of inoculum establishing new foci of infection can greatly increase the spread of epidemics into new areas, whether at the within-field scale or between-fields. Rapid establishment of daughter foci throughout England and Wales has been shown to have greatly speeded the spread of disease in the recent foot and mouth epidemic in the UK (Keeling *et al*., 2001). Each new introduction is surrounded by susceptible hosts, allowing rapid local intensification. Comparable situations occur in many plant diseases. An example is given in Fig. 7 for the spread of rhizomania disease in the UK. The figure shows the results of a stochastic, spatially explicit model identifying three foci of infection (upper right figure) that correspond with reported cases. The sites of primary infection are thought to represent introductions of the disease carried on seed potatoes from continental Europe.

3. *Host Growth and the Dynamics of Disease*

The supply of host tissue undoubtedly affects the dynamics of epidemics, entering into the force of infection from both primary and secondary infection. How does the pathogen affect this supply? Clearly the pathogen renders infected tissue no longer available for infection thereby slowing the epidemic. But can the parasite affect the dynamics of the supply of susceptible tissue more subtly? This is likely to occur when the unit of interest is a leaf or a root since the plant can compensate for low levels of infection by producing more tissue but failing to do so as the load of infection increases. Detection of this effect may be important in the dynamics of epidemics. New susceptibles, produced to compensate for the loss of others, may have two contrasting effects. They may dilute the existing infection allowing greater photosynthesis or nutrient uptake, thereby minimising the effects of infection. They may, however, speed up the epidemic by providing more susceptibles through which the pathogen can spread. This stimulation of the epidemic by the host response to infection load is likely to be important in contributing to spatial spread. Hence the arrival of new susceptible tissue between an infected and a susceptible may act as a 'stepping stone' favouring the spread of disease. Experimental testing of these processes, however, is difficult because of the complicated interactions involved.

Gilligan *et al.* (1997) examined a range of functions to test the effects of host response to infection load on field data for stem canker of potatoes caused by *R. solani* from a factorial experiment with two different levels of initial

Fig. 7. Spatial heterogeneity of disease spread through a heterogeneous mosaic within the landscape. The first three panels show the spread of an introduced disease, *Rhizomania* (large dark dots) of sugar-beet, through farms (small light dots) in East Anglia. Large pale dots represent infested but not yet symptomatic farms. The outbreaks are predicted by a stochastic spatial model. The spread is localised around three initial foci. The other two figures show the result of two simulations for the spread of disease into other sugar-beet growing areas in the UK (light dots represent susceptible farms and dark dots show infested farms). The disease dynamics are highly non-linear and stochastic. From these it may be seen that markedly different scenarios may obtain for identical parameters for intensification, crop susceptibility and transmission, when allowance is made for stochastic variability. (Reproduced with permission from Dr A. Stacey, Epidemiology and Modelling Group, Cambridge: the illustration first appeared in Gilligan (2002)).

inoculum amongst other treatments. The model is a special case of eqn (4) in which there is primary infection but no secondary infection in the spread of stem canker and with an additional class of removed or dead stems. The generic form is given by:

$$\frac{dS}{dt} = r(S,I,X) - Xf_p(S,X),$$
$$\frac{dI}{dt} = Xf_p(S,X) - dI,$$
$$\frac{dR}{dt} = dI.$$

A more precise form is given below. First we decompose the term for host reproduction into two components for host growth in the absence of disease and for a host response to infection load $f(I,S)$:

$$r(S,I,X) = b(\kappa - N) - f(I,S).$$

The net production of stems in the absence of disease is given by a monomolecular function where N is the total number of stems ($N = S + I + R$). The function for host response to infection is given by a wide variety of forms (Gilligan *et al.*, 1997), of which two are summarised here. First is a linear response, $f(I, S) = \alpha I$, in which each additional infected stem causes a proportional reduction in the production of susceptible stems (Fig. 8(a)). The second involves a non-linear response, $f(I, S) = (\alpha_1 I^2 - \alpha_2 I)/(\alpha_3 + I^2)$, in which low levels of infection stimulate the production of susceptible roots and high levels of infection inhibit infection (Fig. 8(b)). Potato stems become resistant to infection over time and the force of infection therefore involves a quenching term, yielding a working model:

$$\begin{aligned}\frac{dS}{dt} &= \left\{b\left(\kappa - N\right) - f(I,S)\right\} - \lambda_0 \exp\left(-\mu t\right)S,\\ \frac{dI}{dt} &= \lambda_0 \exp\left(-\mu t\right)S - dI,\\ \frac{dR}{dt} &= dI.\end{aligned} \tag{5}$$

The parameter λ_0 includes the transmission parameter and the amount of inoculum, which over the time scale of infection was assumed to be constant. In principle, the linear term can be extended by addition of a constant to allow for stimulation at low densities of infection and inhibition at higher densities (Fig. 8(a)). This, however, yields $f(I, S) = \alpha_1 I - \alpha_2$ but the constant term (α_2) cannot be estimated by fitting the model to data because it is subsumed into the other constant term $b\kappa$ for host growth. Extensive fitting of the other models to the field data sets showed that it was difficult to distinguish between models for host response to infection. The closeness of the fit of the two models is shown in

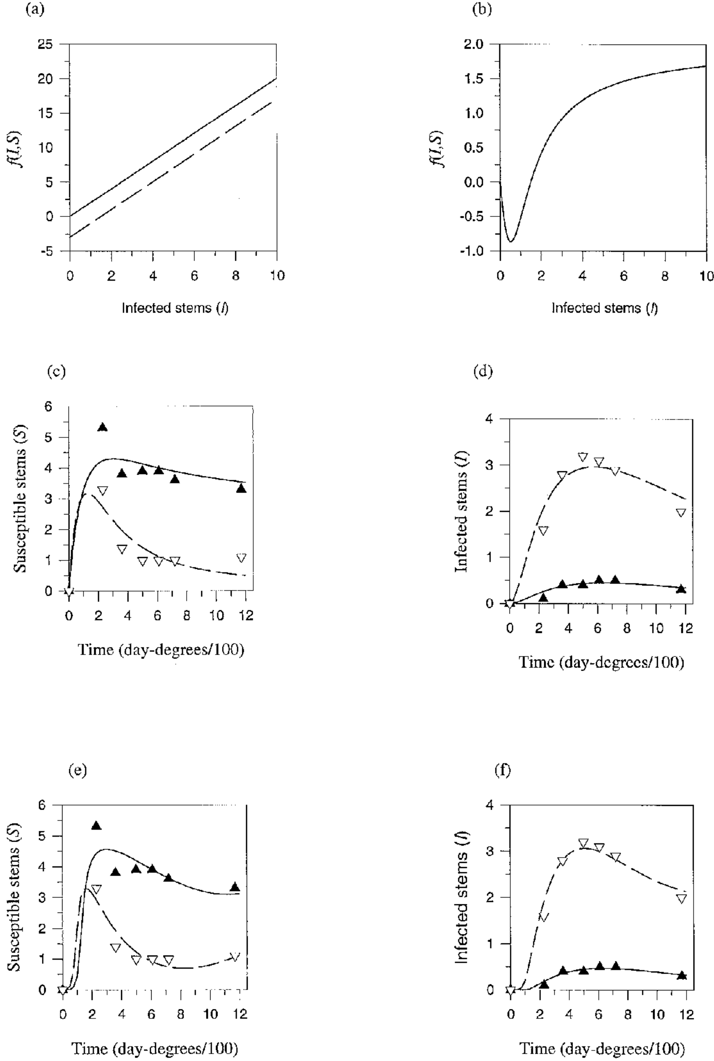


Fig. 8. Illustration of functions for host response to infection load and fit of two alternative models based on eqn (5) to epidemics of stem canker on potatoes caused by *Rhizoctonia solani*. (a) Linear response, $f(I,S) = \alpha I$, in which each additional infected stem causes a proportional reduction in the production of susceptible stems. The dotted line shows a linear response of the form: $f(I,S) = \alpha_1 I - \alpha_2$, with stimulation at low densities but inhibition at higher densities, but this model cannot be distinguished by fitting from the simpler form $f(I,S) = \alpha I$ via model fitting. (b) Non-linear response, $f(I,S) = (\alpha_1 I^2 - \alpha_2 I)/(\alpha_3 + I^2)$, in which low levels of infection stimulate the production of susceptible roots and high levels of infection inhibit infection. (c) and (d) Goodness-of-fit of model with linear host response to field data for stem canker with two levels of initial inoculum. (e) and (f) Corresponding fit of the non-linear model. The data here show little distinction between the two models. Adapted with permission from Gilligan *et al.* (1997).

Figs 8(c) and (e) for two levels of initial inoculum. Fitting did, however, provide evidence for some improved agreement with the data when allowance was made for a host response to infection load. This is likely to be an area of fruitful, if challenging, further study, not least in identifying the effects of small changes induced by environmental or other effects on the early responses to infection. A small change could rapidly switch from stimulation to inhibition of host growth, with quite severe effects on the later epidemic.

4. Linking Within-season Dynamics and Between-season Dynamics

Relatively little thought has been given to the dynamics of inoculum between susceptible crops other than to monitor the decay of inoculum (cf. Wilson *et al.*, 1992). Some pathogens, however, like *R. solani* are capable of saprotrophic multiplication on organic debris. Others, such as *Erysiphe* spp. may grow on alternative hosts. This results in a rise and fall of inoculum during the intercrop period, so that by adjusting the time between harvest and sowing the grower may avoid the peak of inoculum associated with saprotrophic dynamics. Seasonal interruption of host growth introduces a marked discontinuity in population dynamics of many pathogens, but it has been seldom studied. Whereas many epidemiological analyses have considered the effects of seasonal forcing as temperature changes, temporal heterogeneity in the form of disturbance due to sowing followed by harvest has not. This is surprising, since abrupt changes in plant, microbial and invertebrate populations occur not only in annual crops but also in deciduous crops. They also occur with the onset and cessation of favourable and unfavourable conditions for population growth. This switching effect on population dynamics is likely to attract further study of aerial as well as subterranean pathogens. It is particularly important for persistence of pathogens because it imposes differential demands for persistence throughout the crop and intercrop periods in a way that has often been overlooked.

To illustrate this we show first the dynamics of inoculum of *Sclerotinia minor* within and between successive lettuce crops in the presence of a biological control agent, the hyperparasitic fungus *Sporidesmium sclerotivorum* (Adams and Fravel, 1990). This is a three-species system comprising host (lettuce), parasite (*S. minor*) and hyperparasite (*S. sclerotivorum*). This is followed by a brief but more general summary of recent analysis of disease dynamics in the presence of disturbances. The data in Fig. 9 show the status (susceptible, i.e. healthy or infected) of sclerotia of *S. minor* in field plots at Beltsville to which relatively large amounts of inoculum of *S. sclerotivorum* were added. There were five successive lettuce crops, planted in spring or autumn over a period of approximately 140 weeks. There are therefore two epidemics proceeding one on the crop, the other on the pathogen itself: *S. minor* causes lettuce drop disease, while *S. sclerotivorum* is hyperparasitic on *S. minor*. Each epidemic is subject to two sorts of disturbance, one driven by the seasonal forcing associated with marked changes in environmental variables, notably temperature, and the other by pulsed disturbances associated with harvesting and cultivation. One striking

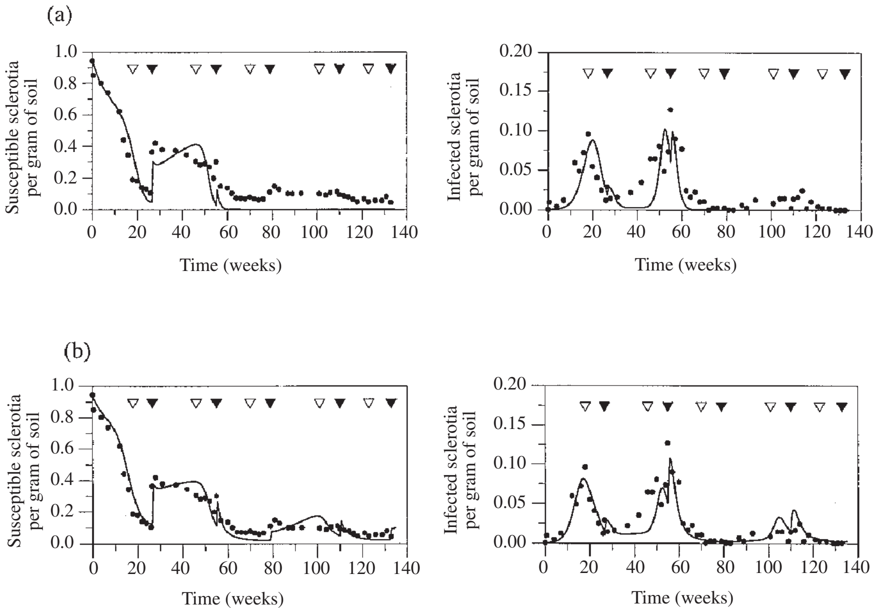


Fig. 9. Fit of two alternative forms of the two species parasite–hyperparasite model to data for the biological control of sclerotia of *Sclerotinia minor* the cause of lettuce drop by *Sporidesmium sclerotivorum* over five successive lettuce crops. (a) Transmission term with simple mixing leads to fade out of susceptible and infected sclerotia. (b) Transmission term with heterogeneous mixing allows persistence. The models incorporated pulsed input of susceptible sclerotia at harvest and seasonal forcing of transmission due to temperature changes. Planting of a crop is marked by a hollow triangle and harvest by a solid triangle. The data were originally collected by Adams and Fravel (1990). Model fits reproduced with permission from Gubbins and Gilligan (1997a).

feature in addition to the pulsed dynamics of *S. minor* is the persistence of both susceptible and infected fractions throughout the period of the experiment (Fig. 9). Hence, although the hyperparasite decreased the amount of parasite present, it did not eliminate it. Numerous questions arise concerning these dynamics but here we summarise three. Is it possible:

1. to capture and reproduce the sudden changes in inoculum density;
2. to prevent fade-out in the dynamics of the parasite and hyperparasite; and
3. to identify the mechanisms responsible for the dynamics?

Gubbins and Gilligan (1997a) explored these dynamics using a range of models with general format *SIX*. Two broad types of model were used, one for three-species dynamics involving the host, parasite and hyperparasite, the other a two-species model for the parasite and hyperparasite. The general form of the two-species model for *S. minor* and *S. sclerotivorum* is given below:

$$\frac{\mathrm{d}S}{\mathrm{d}t} = \left(r_1 + r_2\delta\left(t - T_h\right)\right)S\left(1 - S/\kappa\right) - Xf_s(S,X) - \mathrm{d}S,$$
$$\frac{\mathrm{d}I}{\mathrm{d}t} = Xf_s(S,X) - (d+\mu)I, \tag{6}$$
$$\frac{\mathrm{d}X}{\mathrm{d}t} = gI - hX,$$

in which susceptible hosts are produced by slow release from infected plant material in the soil and from a pulse at harvest time (T_h). Susceptible sclerotia die at a rate dS. Transmission of infection[1] comes by release of spores (X) of *S. sclerotivorum* from infected sclerotia of *S. minor*. Here the disturbance is imposed on epidemics of *S. sclerotivorum* on *S. minor* that otherwise occur continuously through time even when the lettuce crop is absent. The models were analysed with and without allowance for pulsed input, with and without allowance for seasonal forcing in the transmission term, and the transmission term itself was varied to simulate different forms of contact between hyperparasite and parasite (Gubbins and Gilligan, 1997c). This involved replacing a simple mass-action function response in which $f_s(S,X) = \beta S$ with proportional mixing, $f_s(S,X) = \beta C(N)(S/N)$ and with heterogeneous mixing $f_s(S,X) = \beta S^m X^{n-1}$. The following results emerged:

1. without allowance for pulsed disturbance both the parasite and hyperparasite die out too soon;
2. periodic forcing and pulsed inputs affect persistence of the parasite;
3. the transmission term also affects persistence.

Figure 9 shows the performance of two models, one with simple mixing, in which the model fails to capture the persistence and the other with heterogeneous transmission which does. Both models incorporated pulsed inputs associated with harvest and seasonal forcing of the transmission term (Gilligan *et al.*, 1997). The results underline the importance of seasonal disturbance in controlling the dynamics of plant disease and in particular persistence. It is important not only in persistence of the parasite but also in persistence of the hyperparasite for continued effectiveness of biological control as in the *Sporidesmium–Sclerotinia* system. Relatively little thought has been given so far to the deployment of biological control agents. The seminal texts (Baker and Cook, 1974; Cook and Baker, 1983), now quite old, emphasised physiological rather than epidemiological mechanisms. The results described here prompt numerous questions about the nature of the pulse, the differences in dynamics within and between crops, and why should a model that assumes heterogeneous mixing

[1] Despite the superficial mathematical resemblance to primary infection, this is clearly secondary infection, since the rate of transmission depends on the amount of infected tissue. It is therefore driven by the internal dynamics of the epidemic rather than from some 'external' reservoir that characterises primary infection. For clarity, $f_s(S,X)$ could be written as $f_s(S,X(I))$ to show the dependence on I, but this is unnecessarily cumbersome.

succeed in reproducing the data while one with homogeneous mixing fails? The biology of lettuce drop, with its two- and three-species interactions, is rather complicated and may mask some of the governing principles.

5. Generalising Between-season Dynamics

Many epidemics have complicated interruptions that correspond with removal of all or part of a host crop, or where growth of the host ceases. Accordingly, we adjusted the *S. minor–S. sclerotivorum* model to a more general form that applies to a range of epidemic systems having within- and between-season dynamics (Gubbins and Gilligan, 1997b). The within-season dynamics are given by:

$$\begin{aligned} \frac{dS}{dt} &= rS(1 - S/\kappa) - Xf(S,X), \\ \frac{dI}{dt} &= Xf(S,X) - \mu I, \\ \frac{dX}{dt} &= gI - hX. \end{aligned} \tag{7}$$

Three possibilities for between-season dynamics are summarised in Table II. The first type, denoted $N = 0$, disturbance, involves complete removal of the host including infected and susceptible tissue. The second involves removal of healthy hosts, leaving behind previously infected decaying tissue. This is characterised as $S = 0$ disturbance and is typical of many crops in which infected debris remains after harvest. The third type, $r = 0$ disturbance, reflects the case where the host stops producing new susceptibles, existing tissue then decays but some infection is possible as inoculum is released from decaying tissue. Other scenarios can be accommodated, but the three listed here encompass a range of severities of disturbance and hence provide a useful starting point to analyse disease dynamics in the presence of disturbance. Whether or not a parasite can reinvade a crop following interruption by harvest depends upon the amount of infected tissue at the end of the season and the amount of inoculum necessary to initiate an epidemic in a subsequent crop. This suggests that the parasite population must exceed an extinction threshold, X_E, and the density of infected hosts must also exceed a threshold level, I_E, that is sufficient to support the parasite reproduction. Combining these thresholds and the between-season dynamics allows computation of criteria for persistence for each of the three types of disturbance (Table II). Formal treatment of persistence demands consideration of stochasticity and the risk of chance elimination as population numbers become small. This is discussed briefly in Section VI. Here I show that there is a critical period between susceptible crops (T_b) for persistence of disease to occur (Table II). This is given explicitly for $N = 0$ and $S = 0$ disturbance (Table II). There is no explicit solution for $r = 0$ disturbance, but mathematical analysis (Gubbins and Gilligan, 1997b) suggests that there is also a critical interseason period for this case. Future work now needs to explore this framework further and to collate empirical results for persistence, or extinction, of inoculum and disease within this framework.

TABLE II

Examples of three types of epidemic and inoculum dynamics between seasons. The rates of growth of the susceptible and infected hosts and the 'free-living' parasite are shown, together with the critical inter-season period for persistence (T_b). X_n, I_n refer to inoculum and infected density at the end of the nth season (Gubbins and Gilligan, 1997b)

Variable	$N = 0$ disturbance Only the 'free-living' parasite population remains between harvests	$S = 0$ disturbance Infected hosts and 'free-living' parasite remain between harvests	$r = 0$ disturbance Survival but no growth of host between harvests
$\frac{dS}{dt}$	0	0	$-dS - Xf(S,X)$
$\frac{dI}{dt}$	0	$-\mu I$	$Xf(S,X) - \mu I$
$\frac{dX}{dt}$	$-hX$	$gI - hX$	$gI - hX$
T_b	$T_b < \frac{1}{h}\ln\left(\frac{X_n}{X_e}\right)$	$T_b < \min\left(\frac{1}{\mu}\ln\left(\frac{I_n}{I_E}\right), \frac{1}{h}\ln\left(\frac{X_n}{X_E}\right)\right)$	No analytical solution

III. IDENTIFICATION OF EPIDEMIOLOGICAL MECHANISMS: LINKING THE DYNAMICAL CASCADE FROM INOCULUM TO DISEASE

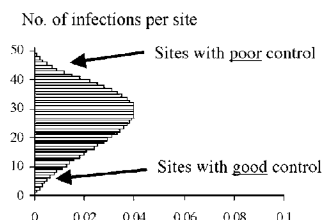
The foregoing analyses illustrate the importance of linking inoculum and disease dynamics. Figure 10 shows schematically how the dynamical cascade from inoculum, through primary and secondary infection, can be used to understand the variability of final levels of disease. The scheme presented here is an amalgamation of experience from more than one pathogen, but serves to show how mechanisms for inoculum and infection can be used to identify mechanisms for control.

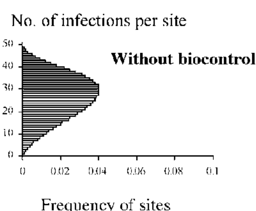
Once a critical parameter is identified, the effects of changing that parameter can be examined using the models. An example is given for the sensitivity of damping-off on radish to r_p (Fig. 11). Knowing the distribution of the variability between epidemics, it is possible to predict how controlling r_p, for example by selecting different isolates of the biocontrol agent, would affect the risk of disease (Gibson *et al.*, 1999). Here we use the probability that a site is infection free, but any other criterion from 0 to 100% infected could have been selected. It is clear that the system is highly non-linear and is highly responsive to very small changes in r_p.

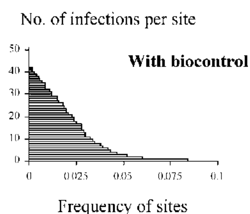
Finding which parameters are governing an epidemic and which are influenced by a particular control treatment is clearly not a trivial exercise. I have shown in section II.B how different models can lead to different inferences, even for microcosm data. Nevertheless the results of these stochastic analyses

1) The efficiency of control can be quantified in terms of risk of disease at different sites using a stochastic model to forecast the frequency distribution of sites with different levels of disease

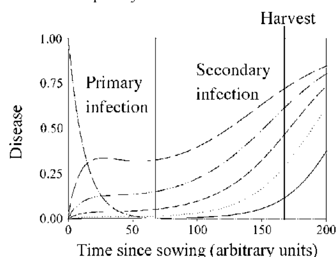
2) This can be done for the presence or absence of control, showing the variability in the final levels of disease and hence the chance of success and risks







3) Final levels of disease depend upon the balance of primary and secondary infection during an epidemic. A small change in the rate of primary infection, even when the rate of secondary infection is unchanged can lead to large differences in the final level of infection and disease. The curves show effects of changing the rate of primary infection, while keeping secondary infection common, on the entire epidemic. The rate of decay of soil-borne inoculum is also shown as the decaying exponential curve.



4) The amount of primary infection in turn depends upon the balance between decay and saprotrophic amplification between susceptible crops. Soil sterilisation and cultural control clearly affect this stage. Biological and chemical control may do so indirectly as the reduced levels of disease or inoculum from the previous crop impact on the carry-over into the inter-crop period.

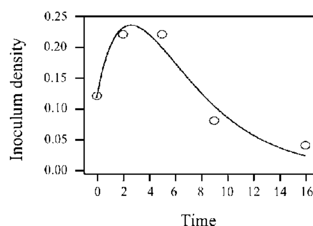


Fig. 10. Schematic representation of the relationships between final levels of disease and some of the epidemiological mechanisms that influence these levels. The data are taken from several soil-borne diseases and are compiled to show the ways that epidemiological analyses can be used to predict efficiency of control and disease risk. Hence changes in carry-over of inoculum may influence not only the mean level of disease at different sites but also the variability and so the risk of disease.

yield methods to compute variability between epidemics and hence risk (Figs 3 and 4). How can this be systematised to improve our understanding of epidemics and disease control? There is a long way to go to advance reliably beyond the empirical but one possible scheme currently under investigation for soil-borne, horticultural, diseases such as damping-off is given in Fig. 12. The method

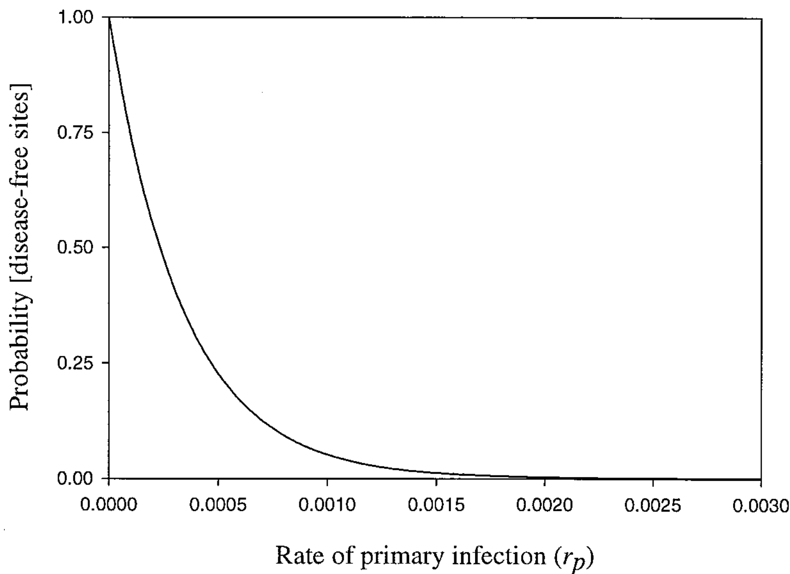


Fig. 11. Variability and disease management. Use of the demographic stochastic model based on equation (2) to consider the effects of selecting control methods with different rates of primary infection (such as isolates of a biological control agent) for the control of disease. Here the criterion is set as the proportion of infection-free sites. Reproduced with permission from Gibson *et al.* (1999).

involves the use of experimental assays to identify which epidemiological mechanisms are influenced by one or more control method. This effect is characterised by a change in parameter value from which it is possible to predict epidemiological risk. Four types of assay are summarised. First is inoculum dynamics, for which modern methods of molecular or immunological detection are improving the feasibility of detection and quantification. Second are pathozone assays. These are individual plant-based assays in which it is possible to characterise the transmission characteristics for primary and secondary infection (Bailey *et al.*, 2000; Stacey *et al.*, 2001) and to scale up from individual to population behaviour (Kleczkowski *et al.*, 1997; Stacey *et al.*, 2001). These lead naturally to microcosm and microplot experiments in which the characteristics of an epidemic are analysed in small populations of hosts from which are derived probability distributions for the evolution of disease over time. The step from microcosms under controlled environments to microplots is important in introducing environmental fluctuations. The advantage of using microplot experiments over large field experiments, especially for soilborne disease, is the feasibility of introducing inoculum at known densities and of monitoring epidemics within relatively small, well-defined sites. The behaviour throughout an entire field can then be inferred by viewing the field as a series of sites in which contemporary small epidemics occur. These may be uncoupled, behaving as distinct, independent patches, with no transfer of inoculum between them as in

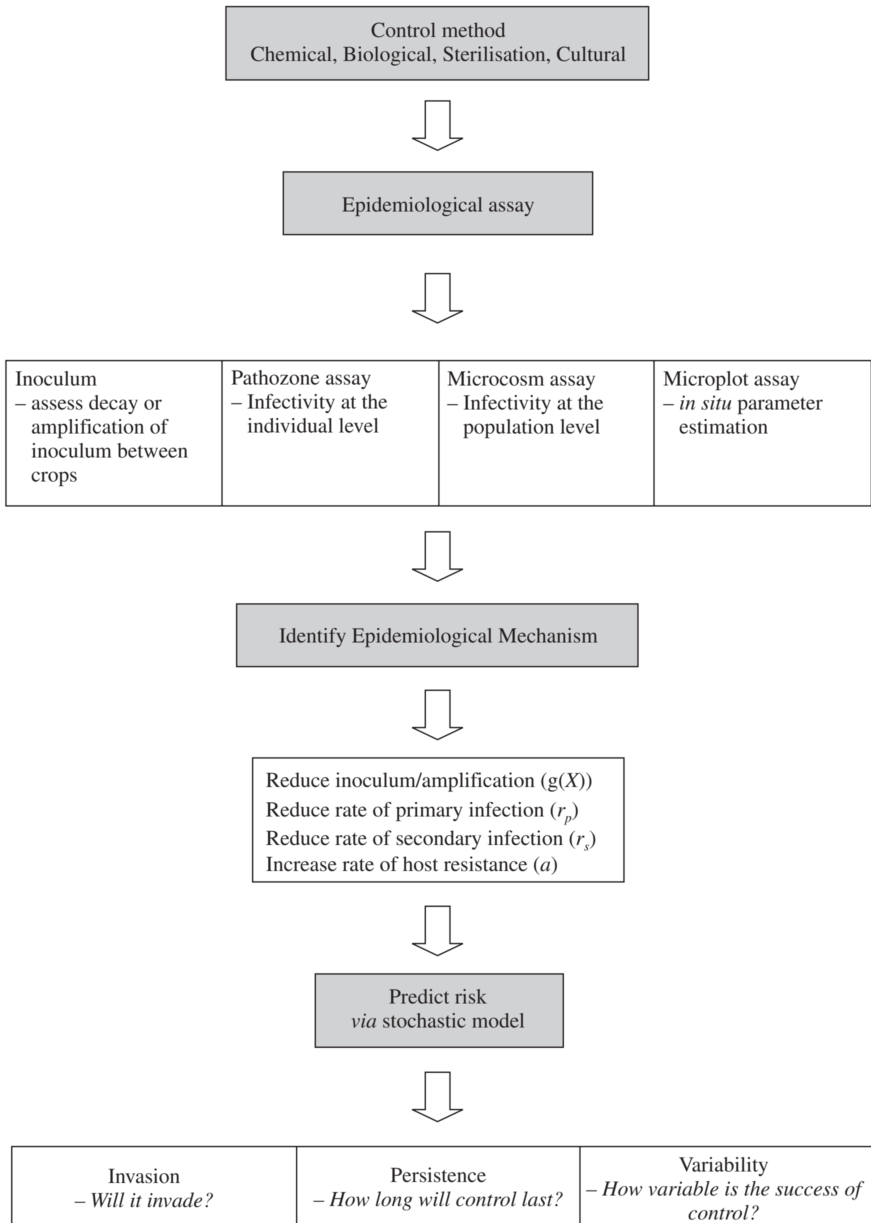


Fig. 12. Schematic representation of the use of epidemiological assays to identify epidemiological mechanisms at the individual and population levels, from which the effectiveness of control and the risk of disease is estimated using a non-linear, stochastic model.

the discussion here. They may be contiguous, perhaps with many unoccupied sites, but with the potential for coupling between sites. This shifts the focus to metapopulations which are discussed in section VII.2.

IV. SIMPLIFICATION OF COMPLICATED MODELS

One of the most important practical questions in epidemiology is to identify which biological processes are controlling an epidemic. This often collapses into a search to show which parameters control the rate of increase or the final amounts of disease. Chemical, biological or other controls can then be deployed so as to optimise the effects on these critical parameters. The problem becomes difficult however, as models become more complicated. It is remarkably easy to elaborate models by including more and more biological and environmental detail, but the cost in loss of transparency into what is controlling the dynamics is severe. It is possible, in principle, to carry out an exhaustive sensitivity analysis in which the effects of changing one or more parameters are examined while holding the others constant. In practice this is usually too costly or prohibitively tedious and some subjective selection is necessary. An example is given in Keeling and Gilligan (2000a) for a human disease, bubonic plague, that persists mostly as epizootics in rat populations (Keeling and Gilligan, 2000b). Here, a model that couples disease and population dynamics in the rat and human populations, each with susceptible, infected and 'removed' individuals, together with the population dynamics of the fleas that transmit the disease, quickly accumulated 18 parameters. This is an alarmingly high number of parameters. Exploration of the model dynamics, showed that it was possible to simplify the biology and hence the model by ignoring the human population and computing instead the potential force of infection to humans. This dropped the numbers of parameters to 11. It is still a relatively large number, but turned out to be sufficiently manageable to show by sensitivity analysis that, of the 11 parameters, only the carrying capacity of fleas per rat had a disproportionately large influence on the potential force of infection to humans. Few models of complicated epidemics are as accommodating. The challenge then is to simplify the model further in order to gain some insight into what is controlling the epidemic. An example is given for the infection of sugar-beet roots by *Polymyxa betae*, the vector of *Rhizomania disease*. The 'epidemiological' dynamics of *P. betae* can be resolved into primary infection from resting spores in cystosori (Blunt *et al*., 1991) and secondary infection from zoospores, while host roots pass through four stages from susceptible, exposed and infected to removed or resistant (Webb *et al*., 1999). The class of infected roots splits into two further types, an *infectious* class that gives rise quite swiftly to zoosporic inoculum and an *infected* class that accumulates resting spores for release after harvest and cultivation. This results, among other things, in two latent periods, one for zoospores and the other for the production of cystosori. The ensuing model therefore has seven compartments, five for the status of sugar-beet roots (susceptible, exposed, infectious, infected and resistant) and two for resting spores and zoospores.

The focus of practical interest is the amplification of resting spores that occurs during each sugar beet crop. Once this becomes large enough, symptoms are expressed in the crop if the fungus is carrying *Beet necrotic yellow vein virus* (BNYVV), the causal agent of *rhizomania*. Only then does the grower become aware of the infestation. The time, measured in years, between arrival and appearance of symptoms is very important for the cryptic amplification and dispersal of BNYVV from the field on agricultural machinery. The amplification of inoculum depends on the accumulation of *infected* root tissue, as well as on the survival of inoculum from previous crops, for which the endoparasitic slime mould, *P. betae* is notoriously persistent. The challenge therefore is to use the information about the biology of the system to predict the amplification of resting spores during the season. The demographic factors affecting the epidemiology and hence the underlying model are therefore rather more complicated than for the system studied by Gubbins and Gilligan (1997b).

Webb *et al.* (1999) showed that the final amount of infected tissue depended on the amount of initial inoculum. Further, rather simple numerical analysis showed how the amount of inoculum at the beginning of a season affected the sensitivity of the amount of infected tissue at the end of the season to each of the 12 parameters. Not surprisingly, large amounts of infected root accumulate when there is a high multiplication rate during the secondary infection phase. However, parameters that increased primary infection also enhanced the production of

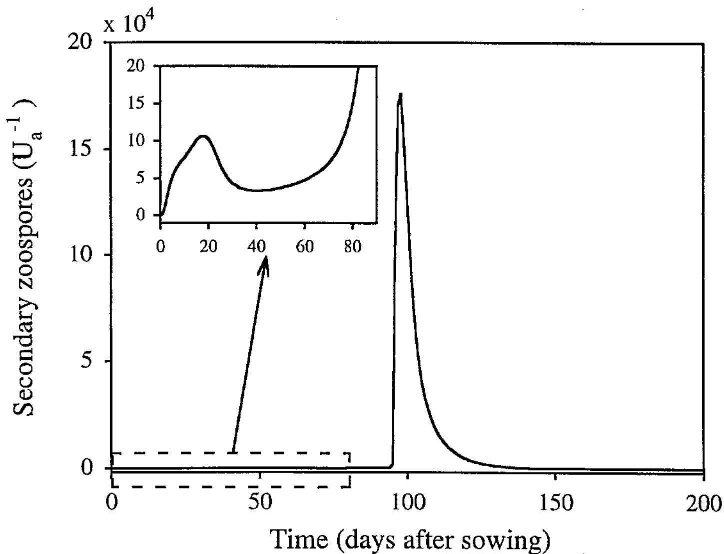


Fig. 13. Temporal dynamics of infection of sugar-beet by inoculum of *Polymyxa betae*, showing that there are two phases of zoospore production, one associated with a short flush due to primary infection and the other with a large flush of secondary infection. U_a^{-1} represents ‘per unit area’. The early flush from primary infection is important in initiating the secondary dynamics. Reproduced with permission from Webb *et al.* (1999).

infected tissue. This is surprising, because although the epidemic resolved itself into two largely sequential phases of primary and secondary infection, these are highly asymmetrical. Primary infection results in a short flush of zoospore production from which a very much larger flush of zoospore production emerges from secondary infection (Fig. 13). The early flush driven by primary infection from resting spores is significant, however, because of the inherent non-linearity of the system. Without this flush the epidemic is unlikely to take off. One striking feature of the system is the relative influence of the two latent periods on the final levels of disease (Fig. 14). High levels of infected root only occur when the latent period for zoospore production (β_1^{-1}) is greater than that for resting spore production (β_2^{-1}). However as β_2 becomes small (and hence the latent period, β_2^{-1}, becomes large) relative to a given value of β_1, the amount of infected root decreases. This occurs because most of the available susceptible sites become filled with zoospores. Once infected, the sites become resistant to infection, reducing the number of sites available for new resting spores.

These examples show that it is possible to extract epidemiologically important information from relatively complicated systems, even when the model is

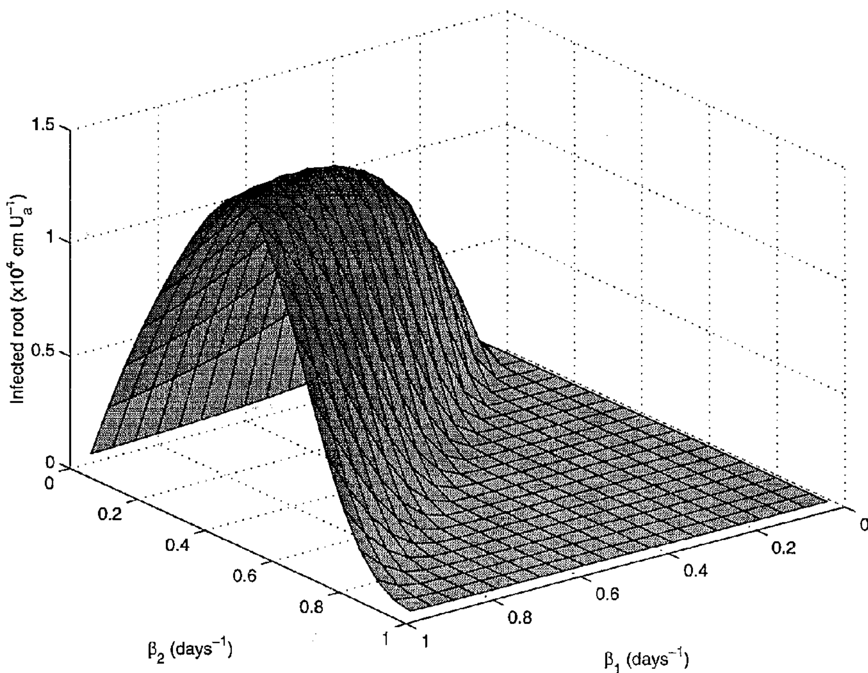


Fig. 14. The effect of varying the relative values of the latent period parameters for infection leading to zoospore (β_1) and resting spore (β_2) production of *Polymyxa betae* on the final amount of infected tissue on sugar-beet. The parameters were scaled relative to maxima, and infected roots are expressed per unit area (U). The figure shows that the relative magnitude of the parameters is crucial in controlling the amount of infected root. Reproduced with permission from Webb *et al.* (1999).

itself relatively complicated. We show elsewhere how to incorporate the effects of temperature on the dynamics of infection (Webb *et al.*, 2000). Of more immediate importance is to find ways of simplifying the model. There is a practical need to identify groupings of the parameters that control the qualitative behaviour of the infection, especially in determining whether or not an epidemic occurs. Ordinarily this is done by derivation of the basic reproductive number, R_0 (see section V) but this is usually derived for systems at equilibrium. Many epidemics do not reach equilibrium, and it is necessary instead to focus on so-called transient behaviour. This is especially important in the sugar beet–*P. betae* system for which the equilibrium levels themselves depend on the initial conditions. Truscott *et al.* (1997) originally showed how to simplify the model when infection was restricted to the relatively simple regime of primary infection. Dealing with epidemics dominated by secondary infection is rather more challenging, but progress can be made towards identifying meaningful groupings of parameters that control the dynamics (Truscott *et al.*, 2000). The approach involves discarding components and processes that do not make a significant contribution to the dynamics of the system. Hence, for plague, it was possible to ignore the detailed dynamics concerning births and deaths in humans for the purposes of estimating the risk to these populations from the faster dynamics of the rat populations. In the *P. betae* system, more formal techniques were used (Truscott *et al.*, 1997, 2000). The analyses show that: although the force of primary infection quickly decays as inoculum is exhausted by initial infections, primary infection acts as a critical trigger for the secondary infection processes. Within the secondary infection phase, there are two regimes: a *quiescent regime* in which the production of secondary zoospores is not sufficient for the cycle to maintain itself without primary input and an *active regime* in which the cascade of secondary cycles is sufficient to maintain the epidemic. There is a critical parameter grouping (Λ) that identifies which regime will be followed. Moreover it is possible to derive approximate expressions to compute the amplification of resting spores during a season. The sign of the critical parameter, Λ, determines which regime the system will attain. This can be recast for $\Lambda = 0$ (Truscott *et al.*, 2000) to give a related criterion,

$$R' = \frac{\text{parameters controlling amplification of infected material}}{\text{parameters associated with loss of infected material}} > 1$$

for amplification. The analyses involve rescaling of the model by nondimensionalisation, followed by asymptotic and perturbation analysis (Murray, 1984; Hinch, 1991) under the assumptions that some parameters are very small or others are very large. Details are given in Truscott *et al.* (1997, 2000). Rough estimates are often available for many of the parameters. For example, it is usually possible to determine how long it takes for a colonised fragment of root to release zoospores or resting spores. Others can be guessed in order to assess the magnitude of R' or Λ and hence to estimate whether or not the epidemic will take off.

Clearly temperature and other environmental variables can affect the dynamics of infection (Blunt *et al.*, 1991). The effects can be translated into temperature

dependence in the parameters (Webb *et al.*, 2000) just as for seasonal forcing of the *S. minor–S. sclerotivorum* system. This accumulates yet more parameters running in the face of model simplification. Alternatively, changes in temperature may be seen to deflect R' above and below one, switching on and switching off the active regime. This may occur at the seasonal level, but it could also be applied, in principle, to daily changes whereby stochastic changes in daily temperature above some critical threshold for microbial infection may switch on and off the epidemic process. This is the subject of continuing research.

V. CRITERIA FOR INVASION

There is already a substantial theory associated with criteria for invasion and persistence in animal and human populations (see the reviews in Anderson and May (1991), Grenfell and Dobson (1995) and Mollison (1995)). Invasion is defined as the introduction and subsequent increase of infection in a host population. Persistence describes a further threshold (Mollison and Levin, 1995) above which the host population provides sufficient susceptibles to maintain both populations so that there is coexistence. Coexistence may mean host and pathogen surviving in natural ecosystems or parasite and hyperparasite in systems involving biological control. In practice, persistence, and to some extent invasion too, is rather more complicated in many botanical epidemics. Mostly these occur in disturbed environments in which the host – or most of it – is removed at harvest or else there is a marked deciduous period, thereby complicating the dynamics of persistence as the pathogen switches between parasitically active and quiescent or even saprotrophically active phases. These were considered in section II.C.5. Here I will concentrate on criteria for invasion during a single growing season.

A. SIMPLE CRITERION FOR INVASION

Ideally, we wish to be able to predict whether or not invasion will occur by knowing something about the epidemiological and host parameters. Hence if we use a partially resistant variety, chemical control or we introduce a biocontrol agent for which we know the effect on disease transmission, we want to determine whether or not the reduction in transmission is sufficient to halt invasion. A little more reflection may lead to a desire to consider the *risk* of invasion that takes account of chance variation between one field and another. It is convenient, however, to start with a deterministic view.

The study of invasion naturally gives rise to the concept of thresholds (Jeger, 1986; Jeger and Van den Bosch, 1994a,b). Invasion thresholds are often related to the basic reproductive number of a parasite, R_0, usually defined as the average number of new infections produced when a single infective individual is introduced into a wholly susceptible host population (Anderson and May, 1979). Whence, for a simple model with logistic host growth and secondary infection:

$$\frac{dS}{dt} = rS\left(1 - \frac{S+I}{\kappa}\right) - \beta SI,$$
$$\frac{dI}{dt} = \beta SI - \mu I, \tag{8}$$

the criterion for invasion is defined as:

$$R_0 = \beta\kappa / \mu \tag{9}$$

that is the product of the transmission rate times the equilibrium density of susceptibles and the infectious period (μ^{-1}). An epidemic is predicted when $R_0 > 1$. Alternatively, a critical population can be defined that is necessary for invasion to occur: $\kappa > \mu/\beta$. The R_0 is analogous to *iR*, of Vanderplank (1963) and related models (Jeger, 1986).

B. MORE REALISTIC CRITERIA FOR INVASION

The models introduced above are restrictive. Further progress involves wider questions:

1. what happens when there are dual sources of infection?
2. does the host response to parasite load affect estimates of R_0?
3. how does heterogeneous mixing affect the dynamics?
4. what does R_0 tell us about risk?
5. what happens in spatially-extended systems where there is sharing of inoculum between fields?

Some of these have been examined in recent work by Swinton and Gilligan, (1999), Gubbins *et al.*, (2000), Madden *et al.*, (2000b), Park *et al.*, (2001) and others. Here, I summarise some results that have applications for management of disease. The reader can find more mathematical detail in the papers cited.

1. *Dual Sources of Infection*

Allowance for primary and secondary infection leads to a simple change in the expressions for R_0 (Gubbins *et al.*, 2000). It emerges that the criterion is a simple sum of components from primary (which we recall may also represent immigration) and secondary infection. Thus, for the generic model given in eqn (4), with no host response to infection, the generic criterion for R_0 is given by:

$$R_0 = \frac{1}{\mu}\left(\frac{g}{h} f_p(\kappa, 0) + f_s(\kappa, 0)\right) > 1,$$

for invasion to occur. We recall that κ is the carrying capacity for stems, roots or leaves or less frequently entire plants of the crop population, g is the parasite reproduction rate, h is the parasite death rate and μ^{-1} is the infectious period. A

specific example given by extending the model in eqn (8) to include a term, $\beta_p XS$, for primary infection clearly shows the additive nature of the result:

$$R_0 = \left(\frac{g\beta_p \kappa}{h\mu} + \frac{\beta_s \kappa}{\mu} \right) > 1.$$

It follows that if the parasite cannot invade by primary or secondary infection alone, invasion may still be possible by the combined processes. This is likely to be important in aerial pathogens in which there may be insufficient inoculum from initial infections to sustain an epidemic, but continued immigration from adjacent or more distant fields may be sufficient to ensure progressive invasion. This additive criterion occurs in related systems where there are dual processes at work, most notably in the biological control of fungal parasites and insect pests, where there are two sorts of transmission, vertical (parent to offspring) and horizontal (between 'parents') of biocontrol agents such as hypovirulence-inducing viruses in Dutch elm disease (Swinton and Gilligan, 1999).

2. *Host Response to Parasite Load and Non-linear Transmission*

Whereas dual sources of infection are relatively easy to analyse, other epidemiological features that affect the inherent non-linearity of epidemics lead to more complicated thresholds for invasion that have only recently been considered (Gubbins *et al.*, 2000; Madden *et al.*, 2000b). Here, I focus on host response to infection and on the nature of disease transmission.

Introducing a simple linear response (section II.C.3) to allow for a simple one-to-one inhibition of susceptible growth does not affect the threshold for invasion. A stimulatory–inhibitory response, $f(I, S) = (\alpha_1 I^2 - \alpha_2 I)/(\alpha_3 + I^2)$, however, does affect the behaviour leading to more complicated thresholds for invasion (Gubbins *et al.*, 2000). There are now three regions of parameter space, which are separated by an extra threshold, R_c with the following outcomes for invasion:

1. $R_0 < R_c$ – no invasion
2. $R_c < R_0 < 1$ – invasion, provided that the infected hosts survive for a sufficiently long time ($\mu < \alpha_2/\alpha_3$)
3. $1 < R_0$ – invasion

It follows that if the parameters that make up R_0 are known, failure to account for a linear host response will make little difference in predicting invasion. But failure to allow for a more complicated relationship in the response of the host to infection could lead to mistakes in prediction. The additional complications arise because of the additional non-linearities introduced by the stimulatory–inhibitory host response. Non-linearities can lead to multiple stable equilibria. For example, when $R_c < R_0 < 1$, there are three equilibria that correspond with different levels of infected leaves or roots:

1. the parasite-free equilibrium, which is stable;
2. an intermediate level of infection, which is unstable;
3. a higher level of infection, which is stable.

This implies that there are also *threshold population densities*, not only of susceptible hosts but of *infected hosts* or *inoculum* for invasion to occur (Gubbins *et al*., 2000). (Although this is obvious to plant pathologists, many of the formal mathematical analyses for invasion strictly hold only for small perturbations to a host crop at equilibrium, because they rely on linearisation around the equilibrium.) Now we can show that a small invading population of parasites may not be sufficient to cause infection above the intermediate equilibrium level and the dynamics slump back to the parasite-free equilibrium, so the epidemic does not take off. A larger invasion, for example by transplanting more infected seedlings, may be sufficient to push infection *above* the intermediate equilibrium level, from which infection proceeds to the higher stable level of infection. The existence of some of these equilibria and how they change according to R_0 is shown in Fig. 15(a) and (b).

Some thresholds are shown in Fig. 15(c) and (d) by taking slices through the S–I and I–X planes. The figures show that there are certain combinations of initial densities of susceptibles, infecteds and inoculum from which invasion is not possible, and other regions from which invasion is possible. The results also show that invasion can occur from either primary inoculum or infected hosts, provided that the initial population exceeds a critical level. Below this value, the parasite is still capable of invading, but only if both sources of inoculum are present initially.

Similar complications arise when we allow for different forms of transmission. Here it is convenient to invoke the functional response to infection and to distinguish the default case of simple linear transmission and to compare it with an accelerating response and a decelerating response. Taking secondary infection as an example, the linear response is given by $f_s(S, I) = \beta_s S$, yielding the familiar mass-action transmission term, $\beta_s SI$. In an accelerating response the transmission rate increases faster than linearly with parasite density, thus $\partial f_s/\partial I > 0$, while for a decelerating response $\partial f_s/\partial I < 0$. Suppose now that the functional response is given by $\beta_s S^m I^{n-1}$(Liu *et al*., 1987; Hochberg, 1991) for heterogeneous mixing. An accelerating response ($n > 1$) can arise when the population is aggregated or if multiple infections are required to cause disease. A decelerating response ($n < 1$) can arise when there is some sort of saturation, perhaps because of competition or because there is incomplete mixing whereby only a proportion of infecteds can transmit infection to the susceptible population. Heterogeneous mixing essentially converts the mean-field model (which assumes that each susceptible plant has an equal chance of being infected by any infected plant, irrespective of where it is in the field) to an implicitly spatial model that allows for enhanced spread between neighbouring plants.

Recent analyses show that a decelerating response does not affect the qualitative behaviour – invasion occurs as for simple mass-action transmission, although there are minor differences in the derivation of R_0, and the quantitative behaviour of disease progress will be different (Gubbins *et al*., 2000). The accelerating response has a major impact, however, on invasion and threshold densi-

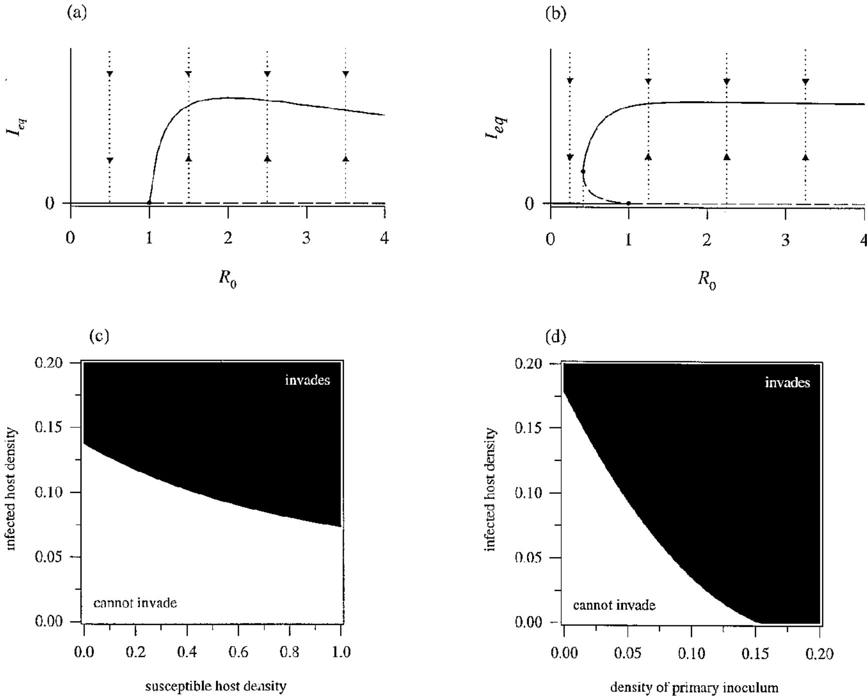


Fig. 15. Invasion thresholds for host–parasite system, given in equation (4) with host responses to infection load, $f(I)$, (a) Mass-action transmission, $f_p\ (S,X) = \beta_p S$ and $f_x(S,I) = \beta_1 S$ with no host response, $f(I) = 0$. (b) Mass-action transmission with a stimulatory–inhibitory host response, $f(I) = (\alpha_1 I^2 - \alpha_2 I)/(\alpha_3 + I^2)$, where $\mu < \alpha_2/\alpha_3$. In the diagrams, a thick solid line indicates an equilibrium that is a node (or focus), a thick dashed line indicates a saddle point, and a dot (•) indicates a change in the patterns of parasite invasion. The arrows indicate the equilibrium to which the populations tend for given initial conditions and, in particular, illustrate the threshold invasion populations for the parasite (the dashed line). (c) and (d) show thresholds in the *S–I* and *X–I* planes, respectively, for invasion. Note that the figures show there are certain combinations of initial densities of susceptibles, infecteds and inoculum from which invasion is not possible, and others from which invasion is possible. Figures adapted from Gubbins *et al.* (2000), with permission.

ties. A criterion analogous to R_0 denoted as σ (Gubbins and Gilligan, 1997b; Gubbins *et al.*, 2000) can be derived for invasion:

$$\sigma \equiv \frac{1}{\mu}\left(\frac{g}{h} f_p\left(S_{\max}, X_{\max}\right) + f_s\left(S_{\max}, I_{\max}\right)\right) > 1$$

The subscript $_{\max}$ denotes the population densities at which the maximum number of new infections per infective unit are produced. Further details and the derivation are given in Gubbins and Gilligan (1997b) and Gubbins *et al.* (2000).

Importantly, the extra nonlinearity because of the transmission term yields two equilibria for $\sigma > 1$, an intermediate one which is unstable and a higher level of infected hosts which is stable. The parasite-free equilibrium is also stable, so small populations of parasites cannot invade. Elsewhere the behaviour is subtle. The ultimate level of infection now depends upon the size of the invading population. Moreover, when there is an accelerating functional response, there is a sharp transition in the relationship between the threshold densities of susceptible and infected hosts. When the initial density of susceptible hosts is high ($S > \kappa/2$) the threshold level for infected hosts is essentially constant. If, however, ($S < \kappa/2$), the critical threshold of initial infected hosts for invasion increases rapidly as the threshold susceptible population decreases. This suggests that there must be enough infection present initially to ensure carry-over until the production of new leaves or roots raises the level of the host population to a level that is able to sustain the epidemic.

Some of these analyses are biologically subtle. The principal results can be summarised as follows: the analyses allow identification of: (1) criteria that apply to invasions during the early stages of crop growth, far away from equilibrium conditions, and (2) threshold populations of susceptible and infected individuals and inoculum that are necessary for invasion. The nature of transmission of infection and the response of the host to infection load – or indeed any other non-linearity in the epidemiological dynamics – may alter profoundly the thresholds. Nevertheless, these results move away from simple systems of parasite-free equilibria to allow estimation of invasion in more realistic agricultural and horticultural environments.

C. APPLICATIONS OF INVASION CRITERIA

Applications of invasion criteria and R_0 in the management of plant disease are still very much at the exploratory stage. Further work is under way in collating estimates for parameters that govern R_0 and in analysis of the functions that translate biological interactions into formal mathematical treatments. We also show below the importance of stochastic variation in the definition and interpretation of threshold criteria. Two likely applications are in gauging the effects of introducing partially resistant varieties and of predicting criteria for the spread of fungicide or pesticide-insensitive forms of parasites. Exploratory work on fungicide resistance (Gubbins and Gilligan, 1999) has shown some promising results with potential for practical applications. By taking a system with two strains, one sensitive and the other resistant to fungicide, it is possible to derive a criterion for invasion that links the effectiveness of chemical control (ε) and the relative fitness of the resistant to the sensitive strain (ρ). The latter is expressed as the ratio of the R_0s for resistant to sensitive, thus $\rho = R_{0\text{res}}/R_{0\text{sen}}$. Invasion occurs when:

$$1 - \varepsilon < \frac{R_{0\text{res}}}{R_{0\text{sen}}} \tag{10}$$

where $\varepsilon = 1$ represents complete control and $\varepsilon = 0$ represents no control. (Note that the convention was reversed in Gubbins and Gilligan (1999), with $\varepsilon = 0$ indicating complete control, but this is less intuitive than the way presented here.) Fungicide efficiency measures the net effect of the fungicide in reducing parasite transmission, parasite multiplication and in enhancing the death rate. The simple criterion in equation (10) applies only when fungicide is continually present. It nicely shows the trade-off between efficiency and relative fitness in determining invasion. Similar criteria have been derived for antibiotic (Austin *et al.*, 1997), drug (Bonhoeffer and Nowak, 1997) and antiviral (Bonhoeffer *et al.*, 1997) resistance. The farmer can do little about the relative fitness of sensitive and resistant forms of the parasite beyond the selection of crop varieties. Fungicide regimes, however, can be changed by using different types of fungicide, and by controlling the amount applied, as well as the number of applications. Now invasion must occur under a regime of periodical change in fungicidal concentration. We could not derive a comparable analytical solution for periodically applied fungicide, but numerical simulations using deterministic and stochastic models show that, for a given level of effectiveness of control, invasion of the resistant form occurs *only* if the relative fitness of the resistant to the sensitive form is sufficiently high (Gubbins and Gilligan, 1999). Moreover, it is possible to show how altering the amount of fungicide applied, and the period between applications, separately affect the probability of invasion and the time to achieve a certain proportion of the resistant form in the parasite population. Hence it is possible to provide rules of thumb for the probability of invasion of the pesticide resistant form. The strategy can be taken further by computing the effects of generation time – to represent different fungal species – and different rates of decay of fungicide – to represent different types of chemical – on the invasion dynamics. Can these theoretical approaches be introduced into practice? Arguably yes, providing that approximate estimates can be made for efficiency and relative fitness. Some equations for the underlying models for host, parasite and chemical dynamics are given in Gubbins and Gilligan (1999). More challenges await, however, in incorporating spatial variation, additional discontinuities as well as interruptions due to harvest, and in considering economic constraints in the invasion of pesticide-resistant (Peck and Ellner, 1997) or other virulent pests and parasites (Gilligan, 2002).

VI. CRITERIA FOR PERSISTENCE: ALLOWING FOR VARIABILITY

A. DETERMINISTIC AND STOCHASTIC THRESHOLDS

The foregoing analyses have focused on deterministic models in order to identify how the mechanisms of disease transmission and host growth can determine whether or not a parasite can invade. The models allow gross distinctions to be made between groupings of parameter values that permit invasion and those that

do not. They do not, however, tell us much about the *risk* of invasion. Deterministic models imply that if R_0 is greater than one, invasion is definite. Clearly this is an oversimplification. Invasion surely depends on chance events in the chain of primary and secondary infections, where, in principle, a few failures at a critical phase may lead to extinction. This may occur because of demographic variability in the probability of transmission, and in the length of the latent or infectious period. It may also be driven by environmental variability, as temperature, rainfall or duration of, say, leaf wetness vary. We are still a long way from a formal treatment of disease risk, but some principles are emerging. Real progress in understanding risk requires a stochastic approach. Here I concentrate on demographic stochasticity, but environmentally driven stochasticity can also be included.

Stochastic models lead, not surprisingly, to thresholds for invasion that have qualitatively similar results to deterministic models demarcating a region of parameter space in which invasion occurs. Three important differences emerge, however:

1. the probability of invasion increases from zero at the threshold, to one further away, as parameters are changed;
2. the stochastic threshold is consistently higher than the deterministic analogue in certain epidemiological systems (Nåsell, 1995);
3. stochastic models also yield a persistence threshold.

Stochastic models therefore allow us to identify the *risk* of invasion and the likelihood of persistence once a parasite invades. The relationships between these thresholds are explored further in section VII. The formulation of stochastic versions of *SEIRX* models follows the general form of eqn (2). Detailed explanations of how to do this for plant disease are given in Park *et al.* (2001) and Shaw (1994). Some recent results using stochastic thresholds to analyse invasion and persistence are given in Swinton and Gilligan (1999) for biological control of Dutch elm disease using d-factors and for fungicide resistance in Gubbins and Gilligan (1999).

B. PERSISTENCE

Persistence of inoculum was introduced in sections II.C.4 and II.C.5 in the context of survival of inoculum between crops. Surprisingly little formal epidemiological attention has been given to persistence in crop disease and the corollary of extinction, yet these underlie many practical issues concerned with the management of disease. Will a new disease, a fungicide-resistant form or a virulent strain, remain in a region, once introduced? How long will it take to eliminate wild-type strains, or will the strains coexist? What is the time to extinction? How long will a biological control agent persist in soil before it is necessary to reintroduce it? Attention may also focus on the fate of the host under threat from a highly pathogenic disease. Elimination of a crop is almost

inconceivable in agriculture, but does apply to annuals and perennials in semi-natural systems. For example, questions have arisen about whether or not the elm will survive following resurgence of Dutch elm disease in the UK (Swinton and Gilligan, 1996). Similar concerns surround the fate of chestnuts in the USA following outbreaks of chestnut blight (Taylor *et al.*, 1998).

Criteria for persistence can be derived by testing for coexistence of disease and the host and then determining whether or not this is stable. This deterministic approach is a useful starting point, but it misses some important features. By failing to allow for stochastic variation, it ignores the risk of fade-out during epidemic troughs, when the infected population becomes very small (Mollison, 1977). Deterministic models, therefore, fail to distinguish between invasion and persistence. If a parasite can invade, then a deterministic model will imply that it can persist. This is not true. Persistence of disease depends on balancing the continued supply of susceptible tissue with the rate of infection. This suggests that there may be selection for intermediate pathogenicity and transmission rates to favour persistence of both parasite and host. If the parasite is too pathogenic with rapid spread then it will quickly exhaust the supply of host and die out. If it is too slow, the crop may outgrow the disease and the parasite dies out, although the evidence for this has yet to be established. The time to extinction of the parasite has been studied in animal and human epidemics (Bartlett, 1956; Grenfell *et al.*, 1995; Swinton *et al.*, 1998), but so far has received little attention in plant disease studies. Bartlett (1956) first showed that there is a critical community size below which the birth rate is too small to support persistent infection. This led to work on the relationship between time to extinction (T_E) and population size (N). A typical expression is given by Ridler-Rowe (1967) as $E(T_E/G) = \ln N$, when there is so called pseudo-mass-action transmission[2] (where G is the mean generation time of the parasite and E refers to the expectation). Barbour (1975) derived a similar relationship with the logarithm of population size, under mass-action transmission, $E(T_E/G) = (R_0/(R_0 - 1)) \ln N + c$ (where c is a constant). The expressions hold for large N. Future applications in botanical epidemiology will undoubtedly encompass spatial considerations, which are discussed below.

VII. INTRODUCING SPACE

Rather like stochastic variation, the spatial structure of epidemics is widely acknowledged for its importance, and then widely ignored! Space can be explicitly included by rewriting the models as partial differential equations (White and Gilligan, 1998) and by the incorporation of dispersal kernels

[2] Here we follow the convention of de Jong *et al.*, (1995) in using mass action to describe a force of infection of the form βSI and pseudo-mass action for the form, $\beta SI/N$. Use of the terms differs among authors, however, and the significance varies in part depending upon whether or not the total population size is fixed and whether S and I are given as densities per unit area or as total numbers (see the review by McCallum *et al.*, 2001 and the response by de Jong *et al.*, 2002).

(van den Bosch *et al.*, 1999). It can also be more easily incorporated in spatially-extended models involving lattices (Gibson and Austin, 1996) or metapopulations. Here I focus briefly on two approaches: percolation and metapopulations. Each allows an intuitive scaling that extends from spread between individual plants to spread between fields. A fuller treatment will be published elsewhere. The questions about disease management that we wish to answer are still focused on invasion and persistence. Will a pathogenic strain invade, how long will it take to invade and how is this affected by the spatial structure of the host crop? How does the mosaic of susceptible fields affect the spread of an epidemic? Are epidemics synchronised within local populations? If disease dies out locally, will it also die out globally? I argue below that spatial considerations can be extended to consider the spread of disease at much larger scales of spread through the landscape. Many of the questions are already in place, some of the theory is known but much still remains to be done.

A. MODEL REDUCTION AND SCALE

A metapopulation typically comprises a set of subpopulations with disease spread occurring within and between subpopulations. The subpopulations may therefore be fields within a region through which disease occurs, with loose coupling (i.e. transmission of inoculum) between neighbouring fields. For some diseases, the metapopulation may occupy the entire field, with subpopulations arising as diseased patches within fields. Lattice systems usually have single plants such as fruit trees at each site (Gottwald *et al.*, 1999). This sort of system naturally leads to consideration of percolation models, whereby there is a critical threshold probability for the transmission of infection between adjacent plants (Grassberger, 1983). Above the threshold there is a finite probability that disease will percolate, i.e. spread while below the threshold disease fails to invade. We have recently shown that this system applies to small-scale dynamics of *R. solani* (Bailey *et al.*, 2000). The two approaches are closely related. Depending on the scale of interest, a metapopulation may be shrunk to a lattice by classifying subpopulations according to whether or not they are uninfected (S), presymptomatic (E), symptomatic and infectious (I) or removed (R). These categories are analogous, though not identical to the *SEIR* format since here we consider symptom expression rather than infectiousness as a distinguishing feature of disease. The spread of disease can now be simulated using an individual-based model such as a probabilistic cellular automaton, with transition probabilities for infection determined by the status of adjacent 'fields' (Keeling and Gilligan, 2000a). Still more simply, each node on the lattice may be classified simply as susceptible (i.e. disease free) or diseased. Ignoring the dynamics within each patch greatly increases the size of the system that can be studied. Thus, we first used a metapopulation model to study persistence of bubonic plague in rat populations, and then a cellular automaton model to study patch dynamics and persistence over very large regions (Keeling and Gilligan,

2000a,b). The approach can readily be adapted to analyse the spread of disease through regions and can be adjusted to allow for gaps in the lattice and random distances between fields.

An example of this approach has been used to study the invasion of rhizomania disease in the UK (Truscott, Stacey and Gilligan, unpublished). The spread of rhizomania occurs over a long period of 15–30 years. It is complicated by cryptic invasion, in which disease is present in crops at presymptomatic levels. This amplifies inoculum in the infested site from which transmission occurs to adjacent fields and farms, principally by movement of infested soil on agricultural machinery (Truscott and Gilligan, 2001). The disease poses several conceptual problems, including the complexity of the epidemiological cycle mediated by the vector, *P. betae*, as well as the importance of presymptomatic spread. Simplification of the model for vector dynamics was outlined in section IV, from which it is possible to compute the amplification of inoculum during each beet crop. Redistribution of inoculum between crops is modelled on a random grid at three scales, within-field, within-farm and between farms, which interact with each other. The model has been successfully fitted to data for the spread of rhizomania in the UK. Some preliminary output is shown in Fig. 7, in section II.C.2. Figure 7 shows the spread of intensification of disease in East Anglia, as well as two stochastic realisations for spread into other regions of the UK. The stochastic realisations underline the possibility of extreme occurrences, shown here as saturated spread or a low incursion. The value of stochasticity lies in assessing the probability of these two contrasting scenarios. Decisions can then be made about the likely effects on the risk of disease spread by the introduction of partially resistant varieties or by disease containment policies.

Still other methods of model simplification involve the approximation of explicitly spatio-temporal models by means of moment closure (Bolker, 1999) and pairwise approximation (Filipe and Gibson, 1998). All of these offer promise for incorporation of the intrinsic non-linearity and spatial structure of epidemics into predictive and management systems. But the theoretical and experimental work is only just beginning.

B. INVASION AND PERSISTENCE IN METAPOPULATIONS

The metapopulation concept is widely used in human and animal epidemiology (Kareiva, 1990; Bolker and Grenfell, 1995; Swinton *et al.*, 1998). With the exception of semi-natural systems metapopulations have received relatively less attention in the study of plant–parasite interactions (Thrall and Jarosz, 1994; Thrall and Antonovics, 1995; Antonovics *et al.*, 1997; Thrall and Burdon, 1997, 1999, 2000; Swinton and Gilligan, 1999; Burdon and Thrall, 1999), despite being a natural scale at which to study the population dynamics (Burdon, 1993; Thrall and Burdon, 1997). This stratification of the host population into a metapopulation can profoundly alter the dynamics of infection, especially the probability of invasion and persistence (Durrett and Levin, 1994b; Levin and

Durrett, 1996). Spatial heterogeneity has recently been the focus of much attention in the dynamics of infection in human and animal populations (Kareiva, 1990; Durrett and Levin, 1994a,b; Swinton and Gilligan, 1998; Swinton *et al.*, 1998). In contrast, the impact of spatial heterogeneity on plant–parasite interactions has received relatively little attention (Onstad and Kornkven, 1992; Thrall and Burdon, 1997; Filipe and Gibson, 1998; Swinton and Gilligan, 1999). Much of the work on epizootic and human diseases is based on deterministic mean-field models, often with assumptions of complete mixing between infecteds and susceptibles, but increasing attention is being given to stochastic (Mollison and Levin, 1995; Nåsell, 1995) and to spatially-extended models drawing on the mathematics of statistical physics, including percolation theory (Durrett, 1995; Cardy, 1996) and probability theory (Grimmett and Welsh, 1986). These approaches are proving a rich ground for analysis of epidemics.

The application of a metapopulation approach to the analysis of invasion and persistence of epidemics of plant disease can be simply illustrated with an example taken from Park *et al.* (2001). Using an *SI* model with density-dependent birth and death of susceptible leaves (or roots), Park *et al.* (2001) derived criteria firstly for invasion in a single subpopulation, here defined as a field, and secondly for a landscape comprising an aggregation of fields on a lattice. The model is given by:

$$\begin{aligned} \frac{\mathrm{d}S_j}{\mathrm{d}t} &= \left(b_0 - b_1 N_j\right) S_j - \left(d_0 + d_1 N_j\right) S_j - \lambda_j S_j, \\ \frac{\mathrm{d}I_j}{\mathrm{d}t} &= \lambda_j S_j - \left(\mu + d_0 + d_1 N_j\right) I_j, \end{aligned} \tag{11}$$

where S_j and I_j are the densities of susceptible and infected hosts (leaves or stems) in the jth field respectively, and $N_j = S_j + I_j$ is the total host density in the jth field. The model for host growth in the absence of disease collapses easily into a logistic function, but, for maximum generality, the birth and death rates are separated here to allow density dependence on both the birth ($b_0 - b_1N_j$) and death ($d_0 + d_1N_j$) rates. The parameter μ is the disease-induced death rate of infected hosts, which, combined with natural mortality, gives a total rate for the loss of infected hosts, as $\mu + d_0 + d_1N_\mathrm{j}$. The fields are linked to allow for transmission from one field to another, with the connections defining the spatial structure of the host population. The force of infection, λ_j depends on two sources of inoculum, most coming from within the field and some coming from neighbouring patches:

$$\lambda_j = \beta I_j + \varepsilon\beta \sum_{k \in N_{\mathrm{hood}}} I_k,$$

where β is the rate of transmission within a patch, and ε is the strength of coupling between fields, that is a measure of the amount of transfer of infection between fields. Here we assume that there is a high production of inoculum, so that the amount of transfer out of the field has a negligible effect on dynamics

within the donating field. Where this is not the case, the equation for force of infection becomes

$$\lambda_j = (1-\varepsilon)\beta I_j + \varepsilon\beta \sum_{k \in N_{\text{hood}}} I_k.$$

The neighbourhood of interaction is a measure of the numbers of fields from which inoculum may arrive in the jth field.

Three key parameters can be identified that play a critical role in the ability of the parasite to invade and persist within the host population (Park *et al.*, 2001). These are:

1. the within-field parasite basic reproductive number (R_p), which characterises the infection dynamics at the local spatial scale within fields;
2. the neighbourhood of interaction (ρ), which describes which fields interact (this may be nearest neighbour, i.e. adjacent fields or may extend beyond nearest neighbours to more distant fields);
3. the strength of coupling between fields (ε) within the neighbourhood of interaction.

The behaviour of the epidemic within fields is characterised by the parameter R_p where:

$$R_p = \frac{\beta\kappa}{\mu + d_0 + d_1\kappa},$$

which is therefore analogous to the now familiar R_0 for an isolated field, with invasion occurring when $R_p > 1$. The parameters, ε, and ρ characterise the spread of the parasite over larger spatial scales. It is not possible to derive an analytical invasion threshold, but a heuristic solution is possible by considering the relative contributions from within and between fields (Park *et al.*, 2001), yielding an R_0 for the entire population of fields of:

$$R_0 = R_p(1 + z\varepsilon)$$

where z is the number of fields in the neighbourhood, which depends on ρ. This, in turn, yields a critical size for individual fields within a metapopulation for invasion to occur ($R_0 > 1$):

$$\kappa = \frac{\mu + d_0}{\beta(1 + z\varepsilon) - d_1}.$$

This is based on an assumption that there is complete mixing between infected and susceptible hosts within a field – an assumption that may well not be true. Nevertheless, these predictions give us a starting point from which to test the model predictions, especially in considering the strength of coupling between fields, which can be controlled by cropping density.

Whereas the deterministic model allows the computation of thresholds for invasion, a stochastic version allows estimation of the *probabilities of invasion*

and persistence. Figure 16 shows how these thresholds are influenced by two of the parameters R_0 and ε, for a given neighbourhood of interaction. The deterministic model separates the parameter space into invasion and non-invasion (Fig. 16(a)). Inspection of the behaviour of the stochastic model around the deterministic boundary shows that although there is a finite probability of invasion immediately above the threshold, the probability of invasion is very low (Fig. 16(b)). Only as the within-field multiplication rate increases substantially does the probability of invasion become large. This is a very important result in considering the risk of invasion. As the value for R_p increases still more, a threshold for persistence emerges (Fig. 16(c)). Spread is now so rapid within fields that the supply of susceptible tissue is rapidly exhausted and the epidemic dies out before there is substantial spread to new regions. This can certainly happen in disease in natural and semi-natural populations (Antonovics *et al.*, 1997; Burdon and Thrall, 1997; Thrall and Burdon, 1999). Its role in agriculture has yet to be assessed and it will be compounded by inter- as well as intra-seasonal dynamics (section II.C.5). It is a feasible consideration, however, in optimising the deployment of control measures involving pesticides, resistant genes, biological and cultural control. How should these be applied across a mosaic of crop sites so as to minimise the risk of invasion and the risk of persistence? Converse problems arise in seeking to establish biological control agents where invasion and persistence of these micro-organisms become desirable.

The stochastic metapopulation theory shows three scenarios: a parasite (or hyperparasite) cannot invade (Fig. 17a); the parasite can invade and persist (Fig. 17(b)); or the parasite invades but cannot persist (Fig. 17(c)). Persistence usually involves local persistence in which the parasite is absent from some fields but present in others (Fig. 17(b)).

The theory allows prediction of the time to extinction. This might be applied to pesticide-sensitive forms and to hyperparasites as for the d-factors to control Dutch elm disease or at a much smaller scale (in which subpopulations comprise sections of a single field) in the use of *S. sclertivorum* to colonise *S. minor.* Persistence clearly depends on the degree of coupling between subpopulations, since these provide the 'rescue-effect' for sites from which inoculum dies out. Park *et al.* (2001) have shown that there are values for the short range transmission (characterised by R_p) and long-range transmission (ε and ρ) that optimise the probability of parasite persistence. Because of the nonlinearity of the system, the outcome depends on the initial levels of infection. Moreover, the chance of extinction is minimised for intermediate levels of coupling. Working with a more general model for metapopulation dynamics, Keeling (2000) has analysed the dynamics of local and global extinctions. He showed that for single-species systems, local extinctions are little affected by coupling, but global extinctions are minimised when coupling is at its maximum. When, however, two species are involved with interdependent dynamics, as for an epidemic, a measure of local extinction increased steadily with coupling between subpopulations, but global extinction was minimised for intermediate coupling.

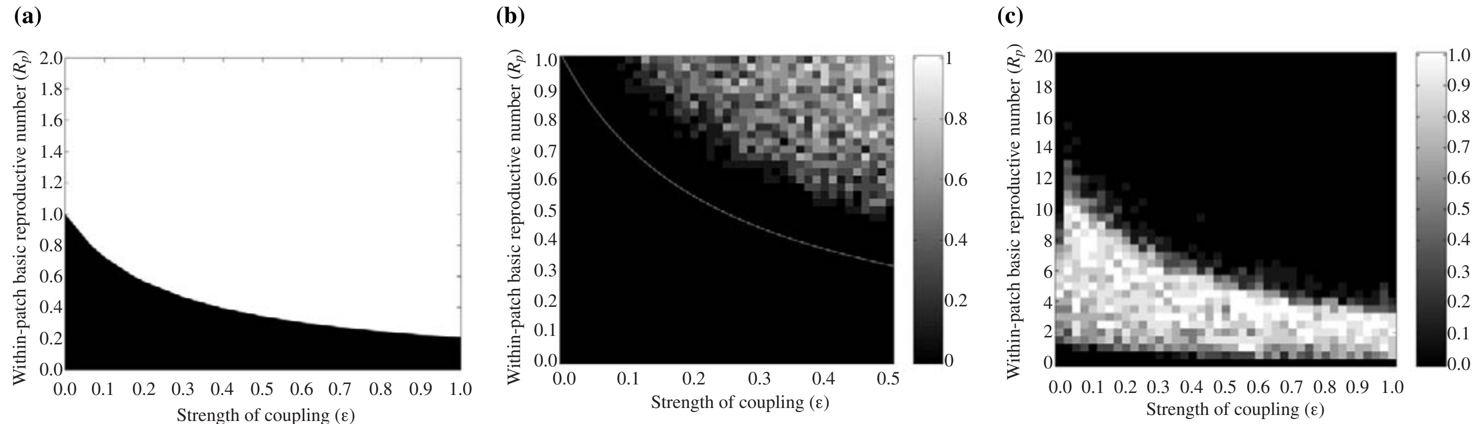


Fig. 16. Comparison of invasion thresholds for a deterministic and a stochastic model for metapopulation dynamics. (a) Invasion thresholds for the deterministic model of disease introduced into a metapopulation. The figure shows how the invasion threshold varies with the strength of coupling between subpopulations ε and the ability to multiply within subpopulations (here denoted as R_p; equivalent to R_0 for a single subpopulation used in the text). The deterministic model implies that the parasite cannot invade in the black region and always invades in the white region. Invasion thresholds also correspond with persistence thresholds: once it invades, a deterministic model predicts that it will persist. (b) Invasion thresholds for the stochastic version of the model. Invasion is now shown as a stochastic process denoted by the grey scale for the probability of invasion, ranging from zero probability (black) to a probability of one (white). The deterministic threshold is shown for comparison. (c) Comparison of invasion and persistence thresholds for the stochastic model. Increasing one of the parameters (R_p) reveals three regimes in the behaviour of a parasite: no invasion (lower black region), invasion *and* persistence (mid region) and invasion followed by elimination (upper black region). The model is a spatially extended generalisation of the simple *SI* model in the text with introduction of a small amount of parasite near the centre of a 10×10 array of subpopulations and allowance for dispersal within and between subpopulations. Reproduced with permission from Park *et al.* (2001).

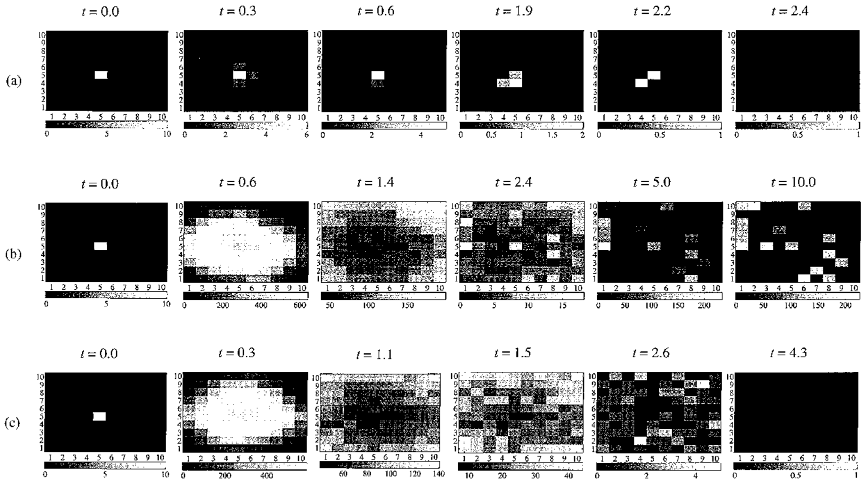


Fig. 17. Three scenarios for the dynamics of an epidemic in a metapopulation. (a) The parasite cannot invade ($R_p = 0.5$). (b) The parasite invades and persists ($R_p = 8.0$). (c) The parasite invades but cannot persist ($R_p = 16.0$). The plots show the infection level in each subpopulation at various times. The bars indicate degree of infection in each subpopulation. The radius of the neighbourhood of interaction is $\rho = 1$, the strength of coupling is $\varepsilon = 0.1$ and the remaining default parameters and initial populations are given in Park *et al.* (2001). Similar results can be derived for systems with more distant dispersal across subpopulations. Reproduced with permission from Park *et al.* (2001).

Future management decisions in epidemiology are likely to consider, not only the deployment of genetical, chemical and other methods of control and their effects on R_p, ε and ρ but also the geometry and size of fields within an agricultural region. Little work has so far been done on the geometry of agricultural mosaics other than in relation to disease mixtures within fields (Mundt and Leonard, 1986; Finckh and Mundt, 1992; Mundt *et al.*, 1995a,b; Mundt, 1997; Finckh *et al.*, 2000; Zhu *et al.*, 2000; Ngugi *et al.*, 2001). An exception is the work of Holt and Chancellor (1999) who have recently used numerical simulations to analyse the spread of rice tungro virus disease in tropical irrigated rice cultivation in which there is asynchronous planting throughout the year. They showed that a relatively large proportion of fields must be planted with resistant varieties in order to have sufficient area-wide impact on inoculum to reduce disease incidence in fields of susceptible varieties. In many rice cropping systems there are two growing seasons per year and the modelling indicated that the best strategy for disease control is to concentrate deployment of resistant varieties in the season of greatest disease spread. Attempts to minimise inoculum carry-over to the 'high-spread' season by concentrating resistant varieties in the previous season had little effect over a range of simulated conditions.

Analytical work on crop mosaics is likely to concentrate upon the size and number of subpopulations. Swinton (1998) has extended the work of Bartlett (1956, 1957) to derive expressions for extinction times. Numerical simulations

show that there is a phase transition (i.e. a switch in behaviour from short to long extinction times) around a critical subpopulation size, N_C. Below N_C, the time to extinction is very short because the amount of susceptible hosts is not sufficient to maintain the epidemic until it spreads to the next subpopulation. As the population of susceptibles increases above N_C there is a sudden transition to long extinction times. Increasing the number of subpopulations in the metapopulation delays extinction but does not affect the critical value of N_C at which the phase transition occurs. The analyses were derived for a rather restrictive type of epidemic associated with the persistence of seal distemper virus in the harbour seal, *Phoca vitulina,* in the North Sea (Swinton *et al*., 1998). This is described by an *SEIR* model with mass-action mixing and nearest-neighbour transmission along a line of subpopulations (typical of a coastline), with a single annual pulse in births of susceptibles. For the current discussion, however, the model serves to draw out the following important points about persistence. Persistence depends on the supply of susceptibles, patch (subpopulation) size, and the number of patches; and there is a critical patch size for disease to persist in a metapopulation. Also there is a marked phase transition towards persistence above the critical patch size. Moreover, the extinction time depends on the time to spread through one subpopulation and the transit time to another subpopulation – defined as the time when the first individual becomes infected and starts an epidemic in the recipient patch.

Swinton also showed that above N_C, the expected extinction time for a metapopulation with $n + 1$ subpopulations each of size N is given by:

$$E\left[T_E\right] = a \ln N + bn,$$

in which a and b are constants. Many botanical epidemic systems appear more complicated with periods of survival on alternative or alternate hosts or by saprotrophy on organic matter, further confounded by genetical and environmental heterogeneity. The phocine distemper virus system also appeared complicated at first sight. Nevertheless the analyses were sufficient to show convincingly that the virus could not persist in the North Sea (Swinton *et al*., 1998). The likelihood of reoccurrence of this devastating epidemic was therefore small, requiring reintroduction of the virus from other species of seals. Metapopulation analyses for invasion, persistence and times to extinction are now an active area of interest in botanical systems.

The metapopulation formulation used by Park *et al.* (2001) is a relatively sophisticated model. It is spatially-explicit, making allowance for stochastic variation, and describes the dynamics of populations within, as well as between, each subpopulation – known as a structured metapopulation (Gyllanberg *et al*., 1997). The original paradigm of a metapopulation arose from work on insect populations by Levins (1969), who defined a population as a series of potential sites for insect colonisation. Each site was either occupied (P) or unoccupied ($1 - P$). This led to the Levins model for metapopulations whereby:

$$\frac{\mathrm{d}P}{\mathrm{d}t} = cP(1 - P) - eP$$

This yields a simple expression for the equilibrium density of occupied patches, $\hat{P} = 1 - e/c$, where c and e are the colonisation and extinction parameters respectively. Surprisingly, this was neither stochastic nor spatial and dealt with one species, yet it has proved remarkably robust in focusing attention on problems of persistence in populations. Recent work by Keeling (2002) on stochastic dynamics for single and interacting species, and by Hanski and Ovaskainen (2002) on explicit spatial structure, have examined the strengths and weaknesses of the simplifying assumption of the Levins model, showing how the model can be used as a rule-of-thumb guide to predict persistence.

C. DYNAMICAL LANDSCAPES

Metapopulations are not static: susceptible crops are rotated. This yields a dynamical landscape through which disease spreads over several years. The motivation for crop rotation has of course been empirical, with farmers quickly realising the risks of disease from repeated monoculture in the same field. Only recently have we begun to think of a theory that underpins this relationship. Initial work will parallel situations of habitat destruction in ecology (which is analogous to harvesting and resowing with a non-susceptible crop) as well as coevolution of hosts and parasites in semi-natural populations, and to the evolution of dispersal. Some general results are emerging: increasing spatial variability leads to selection for reduction in the rate of dispersal, while increasing temporal variability increases the rate of dispersal (McPeek and Holt, 1992). In ecology, paradoxically, the management issue is usually focused on maintaining a race or species in a community, for example by increasing connectivity between favourable patches. By contrast, in epidemiology, we are usually concerned with elimination of the parasite, although future work may seek to promote the persistence of fungicide-sensitive forms in pathogen populations.

Much of the work on landscape dynamics has focused on single species. Some of this can be applied to epidemiological systems under certain conditions, for example,

1. when the amount of host tissue at susceptible sites is not limiting, so that the parasite spreads though a mosaic of susceptible fields;
2. and when sites are simply classified into habitable (susceptible), uninhabitable (unavailable) and habited (infected) without specifying the numbers or infected/diseased status of individuals at each site.

The proportion of the landscape that is available for colonization is of common interest in ecological and epidemiological contexts. This leads to consideration of the total amount of susceptible crop in the landscape, the size and the connectivity of these sites. Seminal work by Fahrig (1992) showed that in ephemeral habitats in which the rate of creation and extinction of patches was fast, the details of dispersal distance and inter-patch distance were relatively unimportant. Habitats for plant disease may be rendered uninhabitable by appli-

cation of pesticide and fungicide as well as by rotation. They are also subject to environmental variation that can effectively switch infection on and off, for example by the availability of moisture for germination. Most environmental switches will act at the global scale, although irrigation is an interesting exception. Hence the agricultural and horticultural landscape may well be ephemeral. Fahrig (1992) did include within-patch dynamics but she also maintained the total amount of habitat as constant. Keymer *et al.* (2000) have taken these analyses a lot further using a combination of mean-field and spatially-explicit models, but without taking account of within-patch dynamics.

Keymer *et al.* (2000) used a patch occupancy model with three states, 'non-habitable', 'habitable and empty', and 'habitable and occupied', to analyse the effects of patch life-span, together with the total amount and connectivity of available habitat on extinction thresholds and persistence in metapopulations. They used a spatially-explicit lattice model with nearest-neighbour mixing, together with a mean-field approximation. The model has four governing parameters, two for patch dynamics and two for biological dynamics.

These are for patch dynamics:

– λ the patch creation rate;
– e the patch extinction rate,

and for biological dynamics:

– β the propagule production rate;
– δ the local extinction rate.

The parameters for patch dynamics were assumed to be independent of location, while the propagule production rate, β, depends on the habitable status of neighbouring patches. In an epidemiological context, β is equivalent to a transmission rate parameter and the whole system can be summarised by an *SIR* mean-field equation, with S equating to empty patches, I to occupied, and R to immune or non-habitable sites:

$$\frac{dS}{dt} = \lambda R - \beta SI + \delta I - eS,$$
$$\frac{dI}{dt} = \beta SI - (\delta + e)I,$$
$$\frac{dR}{dt} = e(S + I) - \lambda R.$$

The long-term amount of habitable sites is given by $\bar{n} = S + I = \lambda/(\lambda + e)$ and the proportion of occupied (i.e. infected) sites is given by $\hat{p} = 1 - (\delta + e)/\beta\bar{n}$, which is analogous to the Levins formula. Ignoring the dynamics of site creation and destruction yields a simple criterion for invasion given by $R_0 = \beta/\delta$. This is a ratio of multiplication (by transmission between sites) and death and is termed the inherent life history of the target species (Keymer *et al.*, 2000). When allowance is made for patch dynamics, a new criterion emerges:

$$R_0^* = R_0 \bar{n} \gamma > 1$$

that depends on the inherent life history, the amount of habitat ($\bar{n}$) and $\gamma = \delta/(\delta + e)$, which is the ratio of intrinsic extinction to effective extinction because of landscape change (Keymer *et al.*, 2000). It follows therefore that it is possible to analyse invasion criteria for disease in the landscape and show how this changes with landscape parameters and intrinsic biological parameters. In particular, it is possible to identify the minimum amount of suitable habitat (n_{min}) that a dynamic landscape needs in order to support disease:

$$n_{min} = \frac{1}{\beta}\left(\delta + \frac{1}{\bar{\tau}}\right)$$

where $\bar{\tau} = 1/e$ is the average life-span of a habitable patch, i.e. the duration of a crop. A corresponding threshold for the minimum life span of a patch can also be derived:

$$\bar{\tau}_{min} = \left(\beta \bar{n} - \delta\right)^{-1}.$$

Of course these results hold only at equilibrium, and the model assumes asynchrony in sowing and harvest, but future work will examine the effects of synchrony. More importantly, the foregoing analyses are based on deterministic analyses. The stochastic version of the models shows that the mean-field (deterministic) model allows an intuitive analysis for invasion that is qualitatively correct. The mean-field model, however, fails to capture the quantitative dynamics of the spread. In particular, the mean-field model grossly underestimates the time taken for colonisation of habitable sites by disease (see figs 2 and 4 in Keymer *et al.* (2000)). Numerical results from the stochastic model also show that the thresholds for R_0^*, n_{min} and $\bar{\tau}_{min}$ are all underestimated if account is not taken of the spatial correlations in local dispersal of the invading species. This highlights the value of the intuition that deterministic analyses allow, while cautioning about the loss of precision and realism that stochastic, spatially-explicit models confer. Without the insight from the deterministic models, however, practical results from simulation may be elusive, remaining hidden in a mass of simulations.

D. PERCOLATION

The connectivity of the habitable sites (fields in which a susceptible crop is grown) is important in determining invasion and persistence of disease in the landscape. This leads naturally to a consideration of percolation, as the rotation of crops – together with the application of pesticides and cultural practices – makes and breaks connections between susceptible and infected crops. Under static landscape conditions, percolation theory predicts that there is a critical probability for connection (p_c) above which the system is connected. Thus, for

the landscape defined above, if $\bar{n} > p_c$ almost the entire landscape will be connected with just a few uninhabitable patches. Below the threshold, habitable patches will exist in isolated fragments. At the notional threshold, theory predicts that the system will be self-similar, having clusters of all sizes from single sites right up to connecting the entire lattice (Stauffer and Aharony, 1991). Hence the critical probability marks a phase transition between isolated sites with a very low probability of invasion below the threshold, while above the threshold the probability abruptly increases. The size of clusters of habitable sites depends on initial conditions. Here percolation refers to the connectivity of the habitable landscape under static conditions, i.e. without crop rotation. In the agricultural landscape, however, invasion and persistence of a parasite depend on the dynamics of the habitat as well as the ability of the parasite itself to percolate through this dynamical landscape – areas of mathematical theory and statistical physics that are still subjects of active research.

Keymer *et al.* (2000) used numerical simulation to examine the rate of change of the landscape, distinguishing three classes: almost static, slow-changing, and fast-changing landscapes. The results are still somewhat distant from most agricultural systems because of the absence of pulsed inputs and perturbations, but they reveal some useful results. Principal among these are:

1. invasion and persistence is possible in dynamic landscapes in which $\bar{n} < p_c$;
2. when the landscape is static and the proportion of habitable sites is high (as for a widely grown susceptible variety), the discrepancy between the mean-field and stochastic, spatial models depends on the transmission and dispersal properties of the invading parasite (encapsulated in R_0), with close correspondence when the invading parasite can spread with relative ease (high R_0), and poor agreement when dispersal is restricted (low R_0).

Hence, failure to take account of landscape changes, brought about by rotation or other agricultural practices, may underestimate the risk of invasion. The analyses show, however, that further progress can be made in planning the deployment of susceptible crops and disease-control strategies, using relatively simple estimates for the ability of some diseases (with high R_0) to spread rapidly through a mosaic of fields. This clearly applies to many wind-dispersed pathogens such as rusts and smuts and those with extensive vector transmission. The feasibility of transmission of soil- and trash-borne diseases by agricultural machinery, however, should not be ignored. Rather surprisingly, little thought has been given so far to the effects of agricultural strategy on the evolution of dispersal strategy among parasites. Travis and Dytham (1999) recently have shown that dispersal rate is generally lowered by reduced habitat availability and by longer habitat persistence. Evolution of dispersal in the agricultural context may occur on a time scale that is long compared with the deployment of resistant varieties. Nevertheless, many fungal parasites do switch between short- and long-distance dispersal when switching between asexual and sexual spore production. The former are often splash dispersed, and the latter frequently

aerially dispersed. Future work will elucidate the relationships of these switches with competition between isolates and landscape dynamics, in order to minimise the risk of severe epidemics.

The concepts underlying percolation are not restricted to a single scale, but apply at all scales. They can be applied to spread between individual hosts. The principle recently has been demonstrated experimentally for the saprotrophic spread of *R. solani,* in which plants (or fields) were represented by nutrient sites comprising agar spots on a triangular lattice. The relationship between probability of colonisation and distance between a donor (an infected site) and a recipient (a susceptible site) was derived from pathozone type experiments (Fig. 18). The distance–colonisation curves were used to estimate the critical inter-site distance (r_c) that corresponded with the critical probability for percolation ($p_c = 0.35$) on a triangular lattice. Hence invasion is expected to occur on lattices with inter-site distances less than r_c, but not on those that exceed the critical distance (Fig. 18). Some results are shown in Fig. 19(a), in which a marked

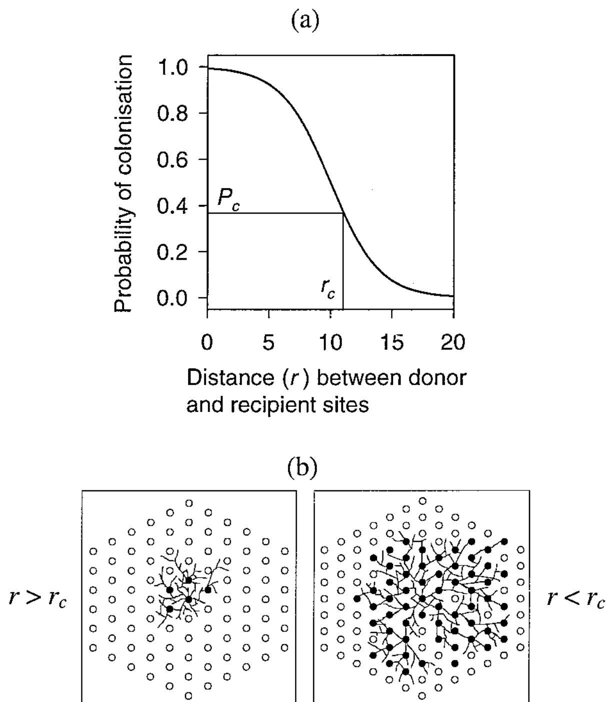


Fig. 18. Use of percolation thresholds to predict whether or not infection invades across a lattice. (a) Quantification of the fall-off in probability of transmission with distance, derived from pathozone-type dynamics involving replicated pairs of donors and recipients, can be used to predict the critical distance (r_c) for percolation to occur in a population. (b) Example of a percolating system ($r > r_c$) and a non-percolating system ($r > r_c$). Adapted with permission from Bailey *et al.* (2000).

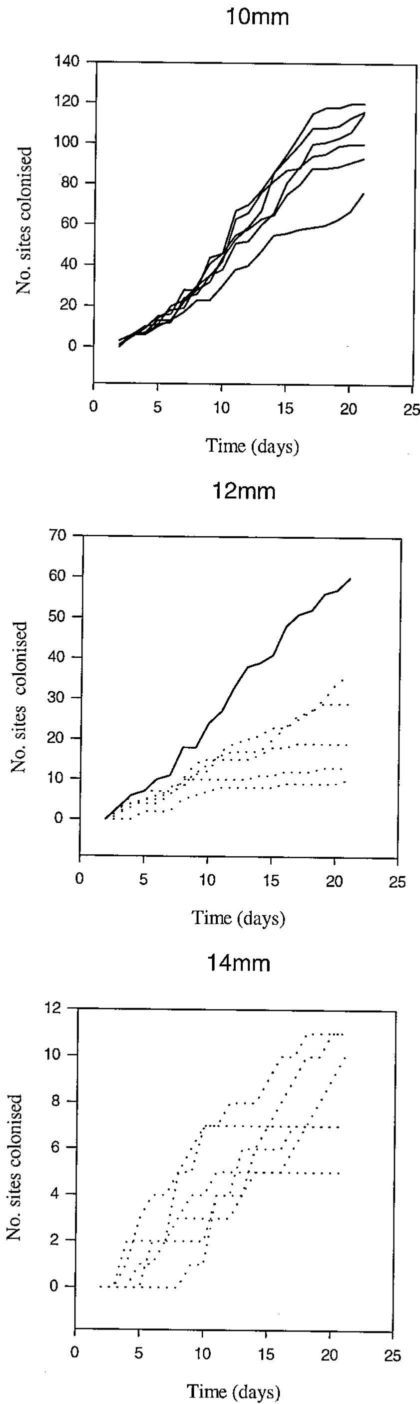


Fig. 19. Effect of critical distance between nutrient sites on a lattice for percolation of *R. solani*. The figures show the change in cumulative number of sites colonised over time. Solid lines indicate invasive spread, and dotted lines indicate finite spread. The critical distance for percolation on this triangular lattice was 11.8 ± 0.93 mm. A sharp transition can be seen between 10 and 12 mm. Data reproduced with permission from Bailey *et al.* (2000).

change in invasive behaviour is seen above and below the percolation threshold distances. The phase transition towards invasion is evident but not absolute, because of the stochastic nature of the process: hence an occasional replicate may invade below the threshold. Reducing the amount of nutrient at each site shifts the transmission curve to the left, thereby reducing the threshold distance (Fig. 19). There are clear analogies here with the planting of crops of differing susceptibilities to disease and the consequence that these have for the critical distance between susceptible crops in the landscape. It is very important to note that the percolation probability dictates the chance of invasion, not the furthest distance that dispersal can occur.

The experimental system used here is simple and rather naïve, but it has the advantage of repeatability. Arguably too, it provides a rigorous test of a theory for invasion and persistence that goes beyond simulation because of the inevitable introduction of some degree of uncontrollable variation in the experimental system.

VIII CONCLUDING REMARKS

This review has emphasised four points in proposing an epidemiological framework for disease management. Simple non-linear models can be used to capture the temporal dynamics of epidemics in a way that enables us to identify criteria for invasion. Variability plays a key role in both invasion and persistence of disease, and can be represented in relatively simple stochastic models. Host growth also affects invasion and persistence, and can be easily incorporated into epidemic models. The models must be elaborated to include spatial variation in order to predict the regional risk of disease.

Much of the work described here has close analogies with other disciplines, notably medical and animal epidemiology as well as metapopulation theory from ecology. Many of the ideas about stochastic and spatially extended dynamics have already been explored in the mathematical and some in the epidemiological literature. Details undoubtedly differ amongst botanical, animal and human epidemics and between the invasion of plant and animal species and pathogenic micro-organisms with their dependence on host dynamics. But many parallels exist, and these have yet to be fully explored. For example, the persistence of plague in rat populations (Keeling and Gilligan, 2000a,b) is analogous to the persistence of a plant disease in an alternative host population or even as a facultative parasite surviving as an active saprotroph. Vectors play an important part in animal and plant epidemics (Chan and Jeger, 1994; Holt *et al.*, 1997). The spatial dynamics of the recent epidemic of foot-and-mouth disease among livestock in the UK (Ferguson *et al.*, 2001; Keeling *et al.*, 2001) resembles many botanical epidemics in which there are two or more levels of mixing, with long-distance transmission initiating new foci and short-distance mixing allowing local intensification of disease (Hughes *et al.*, 1997), for which a rich theory is emerging involving work on small worlds (Watts and Strogatz, 1998; Strogatz,

2001). This theory has important consequences for the spread of resistant races (Fry *et al*., 1992; Milgroom and Fry, 1997), as well as for conventional spread of disease (Zadoks, 1999).

Plant disease is economically important only because it causes crop loss. I have not discussed this at all. Some notable progress is being made on how the dynamics of infection affect crop yield (Madden and Nutter, 1995; Madden *et al*., 2000a). Among a plethora of approaches, most progress is likely to be made in the pursuit of parsimonious models for crop growth (Webb *et al*., 1997) and in exploring the dynamics of disease on the availability of susceptible tissue as well as in the components of yield.

There is a long way to go, but much progress has been made in analysing the spatial and temporal dynamics of disease that underlie invasion and persistence. Providing that we continue to link modelling closely with experimentation and empirical observations, and we guard against the temptation to make models unnecessarily complicated, these nonlinear, stochastic and spatial approaches offer the possibility of improving our understanding of disease risk and so ultimately improving the management of disease at the landscape scale.

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Golgi-independent Trafficking of Macromolecules to the Plant Vacuole

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ABSTRACT

The fidelity of delivery of macromolecules to the plant vacuole is crucial for the maintenance of the essential functions of this organelle. Two different classes of proteins (and

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other macromolecules) can be identified that must be delivered to the vacuole. Firstly, proteins that function in the vacuole are transported there shortly after their biosynthesis, such as seed storage proteins and lytic enzymes. Secondly, proteins that function elsewhere in the cell are delivered to the vacuole for degradation, either in a general homeostatic process for the recycling of damaged cellular components, or in the remobilization of nutrients when under stress conditions. The Golgi apparatus has a major role in the biosynthetic transport of many proteins to the vacuole, and some progress has been made in our understanding of the Golgi-mediated pathways and how proteins are diverted into these pathways from the bulk flow of material to the outside of the cell. However, it is becoming clear that many proteins follow alternative routes to reach the vacuole. Often, this involves the initial assembly of proteins into large aggregates in the endoplasmic reticulum, which then bud into the cytoplasm before transfer to the vacuole. In addition, vacuolar autophagy is emerging as a mechanism for the direct uptake of components from the cytoplasm. Some examples of these pathways and their physiological roles within the plant will be discussed.

I. INTRODUCTION

Plant vacuoles are diverse organelles, both in morphology and function (see Marty, 1999 for review). They are lytic organelles, involved in the breakdown of cellular macromolecules, similar to lysosomes in animal cells. In addition, plant vacuoles have a variety of other essential functions. They are the site of storage of macromolecules, particularly in seeds and in other specialized storage tissues, and of ions and small molecules such as pigments and metabolites. In this role, they are important for cell homeostasis and protection against toxic compounds and stresses. Vacuoles are also required for maintenance of cell turgor, which in turn is a driving force for cell expansion during growth.

Proteins and other macromolecules are transported to the vacuole by a variety of different mechanisms. Many resident vacuolar proteins are transported via the endomembrane system, either by the extensively studied Golgi-mediated pathway, or in vesicles budding directly from the endoplasmic reticulum. In addition, cytoplasmic components are delivered to the vacuole for degradation, in particular under stress conditions. In this review, I will focus on some of the pathways for the Golgi-independent delivery of macromolecules to the plant vacuole.

II. TRANSPORT OF PROTEINS TO THE STORAGE VACUOLE DURING SEED DEVELOPMENT

Most resident vacuolar proteins that have been studied are synthesized at the endoplasmic reticulum on membrane-bound polysomes, and are co-translationally inserted into the endoplasmic reticulum (ER) due to the presence of an ER signal sequence at the N-terminus of the protein. From the ER, multiple different pathways exist for the further transport of these proteins through the secretory pathway. The best studied of these pathways in plants and other organisms are the Golgi-mediated transport pathways. Transport vesicles budding from the ER

carry proteins to the *cis* face of the Golgi, where the vesicle and Golgi membranes fuse, releasing the cargo into the lumen of the Golgi. Proteins move through the Golgi stack, either by vesicle trafficking or cisternal maturation, to the *trans*-Golgi network (TGN). Here, proteins destined for the vacuole are sorted away from secretory proteins, which are secreted to the outside of the cell by a default pathway. Many vacuole-targeted proteins contain a vacuolar sorting signal that is recognized by a TGN-localized sorting receptor. The receptor, along with its bound cargo, is packaged into transport vesicles for transport on to the vacuole, probably via a prevacuolar compartment (for a review, see Bar-Peled *et al.*, 1996). The situation is complicated by the presence in some plant cell types of multiple kinds of vacuole coexisting within the same cell, requiring distinct sorting signals for correct protein targeting (Paris *et al.*, 1996; Swanson *et al.*, 1998; Jauh *et al.*, 1999). It is thought that proteins containing an N-terminal sorting signal are transported to lytic vacuoles in clathrin-coated vesicles (Fig. 1A), and proteins with a C-terminal vacuolar sorting signal are transported in smooth dense vesicles to storage vacuoles (Fig. 1B; Kirsch *et al.*, 1994; Hohl *et al.*, 1996; Hinz *et al.*, 1999; Hillmer *et al.*, 2001). However, at least in most cases, the proteins transported by these pathways pass through the Golgi apparatus.

Several Golgi-independent biosynthetic transport pathways to the vacuole have now been described in various plant species. Typically, these pathways have the common feature of accumulating the protein to be transported in the ER, visualized as electron-dense inclusions by electron microscopy. Budding of the membrane surrounding the protein to form free vesicles is followed either by fusion of the vesicle with the vacuole, or uptake by vacuolar autophagy.

A. TRANSPORT OF SEED STORAGE PROTEINS BY VACUOLAR AUTOPHAGY

During seed development, massive quantities of seed storage proteins are synthesized and transported to protein bodies or to protein storage vacuoles. This transport can take place by several different routes, depending on the species and the protein being transported. Seed storage proteins contain an N-terminal signal sequence for transport across the endoplasmic reticulum membrane into the lumen during synthesis. From this point the transport pathways of different proteins diverge, due to the presence of targeting signals in the protein and the physical properties of the storage proteins themselves. Some storage proteins are transported to the vacuole by the classical Golgi-mediated route, described in Fig. 1. A second class of proteins aggregate in the ER to form protein bodies that eventually detach from the ER cisternae and remain in the cytoplasm (Fig. 2A; Herman and Larkins, 1999).

Of particular interest is a third class of seed storage proteins, for example, the wheat prolamins. These prolamins assemble in the ER, as above, to form protein bodies, surrounded by rough ER, which bud into the cytosol. The small cytosolic

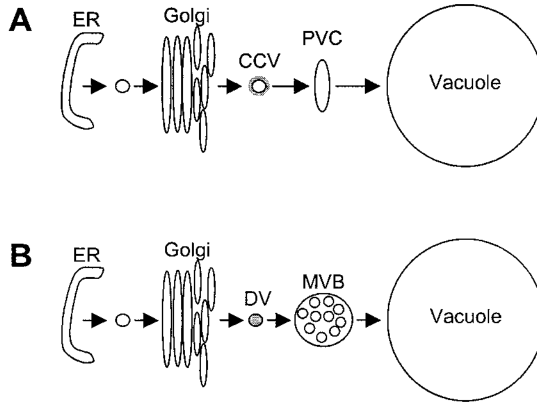

Fig. 1. Golgi-mediated vesicle transport pathways to the vacuole. (A) Many proteins destined for the lytic vacuole travel from the ER to the Golgi and then to the *trans*-Golgi network. Here, they are packaged into clathrin-coated vesicles (CCV) for transport to the vacuole, probably via a prevacuolar compartment (PVC). (B) Storage proteins are often transported from the Golgi to the vacuole in smooth dense vesicles (DV), without a clathrin coat, potentially via a multivesicular body (MVB) that may function as a prevacuolar compartment.

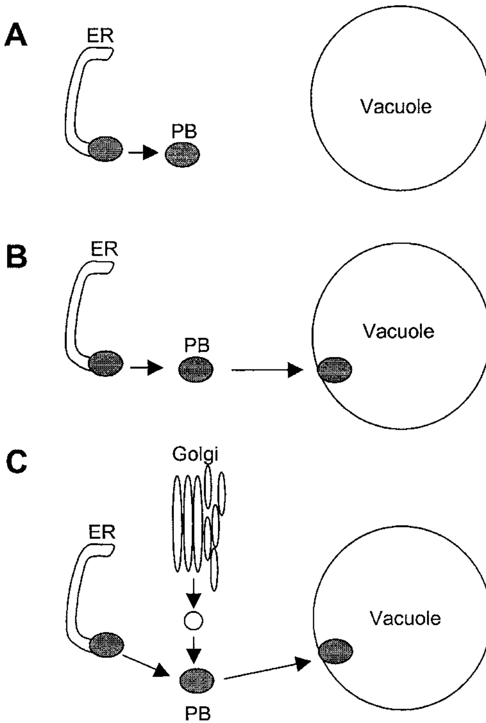

Fig. 2. Formation of ER-derived protein bodies. (A) Protein bodies (PB) form by accumulation of seed storage proteins in the ER, followed by release into the cytoplasm. (B) In some species, the protein body membrane then fuses with the tonoplast, releasing the storage proteins into the vacuole. (C) Protein bodies may acquire additional proteins, containing complex glycans, by the fusion of vesicles derived from the Golgi apparatus.

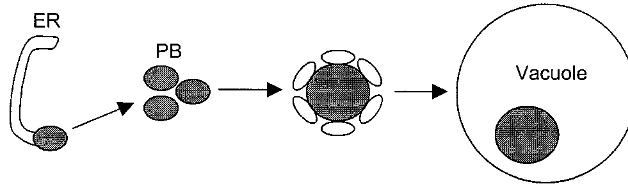

Fig. 3. Vacuolar uptake of protein bodies by autophagy. ER-derived small protein bodies (PB) can fuse in the cytoplasm to form larger aggregates. These become surrounded by membrane vesicles that fuse to form small vacuoles containing the storage proteins.

protein bodies appear to fuse in the cytosol to form larger protein bodies that are often still associated with rough ER membrane. The ER chaperone BiP is present in the protein bodies, confirming their ER origin. However, these protein bodies are then taken up into small vacuoles (or provacuoles) by a process resembling autophagy, at least morphologically. The pathway for this internalization has been studied by electron microscopy (Levanony *et al.*, 1992). Electron-lucent vesicles, or provacuoles, appear to attach to the surface of the cytosolic protein bodies, eventually covering the entire surface. Fusion of the membranes of the provacuoles results in formation of a small vacuole containing one or more protein bodies. These protein bodies initially are surrounded by membranes, which may be derived both from the ER and from the provacuoles. This membrane is apparently degraded within the vacuoles, leaving the protein bodies free inside the vacuole, where they may aggregate further (Fig. 3).

The direct transport of prolamins to the vacuole by autophagy, bypassing the Golgi apparatus, is further supported by studies of the expression pattern of genes involved in protein trafficking (Shy *et al.*, 2001). During wheat endosperm development, a gene required for ER protein translocation (Sec61α) is upregulated as expected, due to the large amounts of storage proteins that need to be transported into the ER. In contrast, two genes involved in trafficking at the Golgi complex (COPα and BP-80) show reduced expression during seed storage protein deposition. These experiments provide correlative evidence that the Golgi complex is not involved in transport of seed storage proteins in wheat.

B. TRANSPORT OF STORAGE PROTEINS IN PRECURSOR-ACCUMULATING VESICLES

A potentially similar pathway for deposition of seed storage proteins has also been described in developing pumpkin cotyledons and castor bean seeds. Initially, the transport and processing of storage proteins was studied by pulse-chase analysis followed by cell fractionation (for example, Fusikawa *et al.*, 1988; Hara-Nishimura *et al.*, 1993). Dense vesicles were identified as a transport intermediate between the rough ER, where the storage proteins are synthesized, and the protein storage vacuoles, where final processing of the proproteins

occurs to produce the mature form. As could be seen for the wheat prolamins, electron-dense aggregates of the storage proteins were observed in the ER, indicating that this may be the source of the vesicles. The dense vesicles were purified, and shown to contain the proforms of seed storage proteins, again indicating that they are an intermediate in the vacuolar transport pathway (Hara-Nishimura *et al.*, 1998) and hence were named 'precursor-accumulating (PAC) vesicles'.

Ultrastructural analysis of both the purified vesicles and pumpkin developing cotyledon cells showed that the vesicles share a number of characteristics with the ER-derived protein bodies of wheat described above. The PAC vesicles are large, approximately 300 to 400 nm in diameter, and appear to form in the rough ER and bud into the cytosol. Some cytosolic PAC vesicles could still be seen in association with ER membranes and ribosomes, and BiP was found within the storage protein aggregates. The purified vesicles consisted of an electron-dense storage protein core, surrounded by an electron-lucent layer that sometimes contained small electron-lucent vesicles. These structures could correspond to the provacuoles described in maturing wheat seeds. In castor bean seeds, this peripheral area of the PAC vesicles also contained glycoproteins containing complex glycans that are formed in the Golgi apparatus (Hara-Nishimura *et al.*, 1998). It is possible that Golgi-derived vesicles fuse with the ER-derived cytosolic PAC vesicles to deliver complex glycan-containing proteins (Fig. 2C).

While these properties suggest that protein bodies in wheat and PAC vesicles in pumpkin and castor bean are in fact equivalent structures, it is unclear how transport of the seed storage proteins from the PAC vesicles to the protein storage vacuoles actually occurs. It is tempting to speculate that, by analogy with the wheat system, PAC vesicles are taken up into the vacuole by an autophagy-like mechanism (Fig. 3). However, it is also possible that storage proteins are delivered by fusion of the outer membrane of the PAC vesicles with the tonoplast of the protein storage vacuole (Fig. 2B,C). Resolution of this question awaits further details on the mechanism of transport.

In addition to seed storage proteins, PAC vesicles have also been shown to transport membrane proteins to the vacuole. One seed-specific component (MP73) was isolated as a peripheral membrane protein from pumpkin protein storage vacuoles (Mitsuhashi *et al.*, 2001). The protein is synthesized as a preproprotein, with an ER-targeting signal and a propeptide of unknown function that is removed in the vacuole. The pro-form of MP73 was found on the membranes of PAC vesicles, whereas the mature form was found at the protein storage vacuole, strongly implicating the PAC vesicles in the transport of MP73 from the ER to the storage vacuoles. PAC vesicles (and probably their equivalent in other species) are thus involved in the delivery of other proteins, in addition to seed storage proteins, that function at the protein storage vacuole.

A second class of membrane proteins found in PAC vesicles are those homologous to proteins involved in vesicle trafficking and protein targeting in various species. Vacuolar sorting receptors from several plant species have been

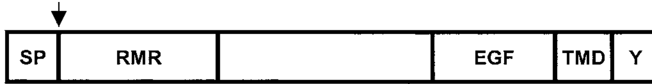

Fig. 4. Structure of vacuolar sorting receptors. Vacuolar sorting receptors contain an N-terminal ER signal peptide (SP) that is removed during synthesis (arrow). The long lumenal portion includes an RMR domain and three epidermal growth factor (EGF) repeats. A single transmembrane region (TMD) is followed by a C-terminal cytoplasmic tail containing a tyrosine (Y) motif for clathrin adapter binding.

described, sharing extensive sequence similarity, the best characterized being pea (BP-80; Paris *et al.*, 1997) and *Arabidopsis* (AtELP; Ahmed *et al.*, 1997). They are integral membrane proteins with a large lumenal domain that binds to N-terminal vacuolar sorting signals from a variety of proteins (Fig. 4). At least in pea and *Arabidopsis*, they reside on the *trans*-Golgi network, where they bind their vacuolar-targeted cargo and package it into clathrin-coated vesicles for further transport on towards the vacuole (Kirsch *et al.*, 1994; Sanderfoot *et al.*, 1998; Cao *et al.*, 2000). Unexpectedly, a membrane protein (PV72) showing sequence homology to these vacuolar sorting receptors was identified in PAC vesicles (Shimada *et al.*, 1997). PV72 was able to bind to peptides derived from pro-2S albumin, implicating it in the recognition and targeting of 2S albumin to the protein storage vacuole. PV72 may therefore act as a sorting receptor for seed storage proteins in pumpkin. One possibility is that PV72 binds to storage proteins in the ER and initiates their aggregation to form the electron-dense core of the budding PAC vesicles. This observation extends the role of this family of vacuolar sorting receptors from Golgi-mediated transport to the vacuole in clathrin-coated vesicles, to a potential function in other types of vacuolar trafficking pathway. Multiple homologs of BP-80/AtELP exist in various species, and it will be interesting to determine whether some of these proteins also function in pathways other than the classical Golgi-dependent pathway.

The association of PV72 with 2S albumin peptides was recently examined in more detail (Watanabe *et al.*, 2001). Like other sorting receptors, PV72 contains epidermal growth factor (EGF)-like repeats in its large, N-terminal lumenal domain. The PV72 lumenal domain, and a truncated version lacking the EGF repeats, were expressed in insect cells and purified for use in binding assays. It was determined that the EGF repeats are not required for binding to the 2S albumin peptide, but appear to stabilize PV72 ligand binding. It has been proposed previously, for other vacuolar sorting receptors studied (Kirsch *et al.*, 1994; Ahmed *et al.*, 2000), that binding of a cargo protein to its receptor is pH dependent. Vacuolar cargo proteins would bind to the receptor at the more neutral pH of the Golgi complex, and be released later in the pathway, possibly in a prevacuolar compartment, due to the more acidic pH of later organelles. In contrast, association and dissociation of the 2S albumin peptide with PV72 was dependent not on pH, but rather on the calcium ion concentration (Watanabe *et al.*, 2001). This may reflect differences in the transport pathway, as PAC

vesicles transport proteins to the protein storage vacuole, which has a more neutral pH than the lytic vacuole. Transport of storage proteins via PAC vesicles may therefore be a calcium-regulated process.

The membrane of PAC vesicles also contains two members of the rab family of small GTPases (Shimada *et al.*, 1994). Rab GTPases are typically involved in vesicle fusion reactions throughout the endomembrane system and may impart specificity to the reaction and regulate membrane fusion via their GTPase cycle. Their presence on PAC vesicles implicates the participation of these membranes in a vesicle fusion reaction. This may indicate that PAC vesicles do in fact fuse with the protein storage tonoplast, rather than being taken up by autophagy. However, the GTPases could also play a role in other types of membrane fusion processes. For example, the membranes of the electron-lucent vesicles surrounding the core of the PAC vesicles may fuse with each other to generate small provacuoles containing the storage proteins.

C. BIOGENESIS OF COMPARTMENTS WITHIN THE PROTEIN STORAGE VACUOLE

The transport of seed proteins to the storage vacuole is complicated by the observation that, in many species, the protein storage vacuole is composed of three distinct compartments. The matrix is the destination for soluble storage proteins, the globoid contains phytic acid and oxalate, and the crystalloid is a compartment defined by its lattice structure and contains a mostly unknown complement of proteins and membrane components. The biogenesis of this unique structure has now begun to be addressed.

In developing tobacco seeds, a transgenic reporter protein was found to localize to small organelles in the cytoplasm at early stages of development (Jiang *et al.*, 2000). These organelles were suggested to be the equivalent of PAC vesicles and to contain material budding directly from the ER, as well as Golgi-derived components (Fig. 2C). Later in seed development, protein storage vacuoles appeared to take up these organelles, which then aggregated to form the crystalloid.

These same PAC vesicle-like organelles were also postulated to be a prevacuolar compartment for the globoid cavity. Jiang *et al.* (2001) demonstrated that the globoid cavity is a membrane-bounded compartment within the protein storage vacuole, which contains membrane and soluble protein markers characteristic of a lytic vacuole. This raises the intriguing possibility that this provides a means of separating lytic and storage functions of the vacuole during seed development. Whether the contents of the globoid compartment are released during seed germination and function in the breakdown of the seed storage proteins in the matrix of the protein storage vacuole remains to be seen.

D. INDUCTION OF ER-DERIVED PROTEIN BODIES IN TRANSGENIC PLANTS

While the process of autophagy during seed development might appear to be unique to certain species, evidence indicates that the pathway may be induced in

species that do not normally produce ER-derived protein bodies (Bagga *et al.*, 1995, 1997; Coleman *et al.*, 1996). Expression of zeins (members of the prolamin class of seed storage proteins) in transgenic tobacco or alfalfa plants caused the formation of protein bodies in the ER that were subsequently transported into vacuoles in seeds by autophagy. The autophagic machinery must therefore be present in species that do not usually synthesize vacuolar protein bodies. Whether this machinery is related to that required for stress-induced autophagy is unclear at this time.

ER-derived protein bodies were also generated in transgenic plants expressing a modified version of the vacuolar seed storage protein vicilin, containing a C-terminal KDEL motif (Wandelt *et al.*, 1992). KDEL (K = lysine, D = aspartate, E = glutamate, L = leucine) motifs are retrieval signals found at the extreme C-terminus of soluble resident ER proteins (Munro and Pelham, 1987; Lee *et al.*, 1993). Upon escape of a KDEL-containing protein from the ER, the KDEL is recognized by a receptor protein at the *cis* Golgi, causing recycling of the protein back to the ER. Addition of a KDEL to vicilin would therefore be expected to greatly reduce its rate of transport to the vacuole and increase its concentration in the ER. Vicilin-KDEL accumulated to very high levels in transgenic plants, compared with unmodified vicilin, and was found to be much more stable. Protein bodies formed in the ER of the vicilin–KDEL-expressing plants and eventually budded off the ER to form protein bodies in the cytoplasm (Wandelt *et al.*, 1992). However, it is not clear whether these protein bodies were finally transported to the vacuole by autophagy, as might be expected, at least in seeds.

Similarly, Hayashi *et al.* (1999) expressed in *Arabidopsis* a fusion protein containing 2S albumin from pumpkin, which normally is transported to the vacuole via PAC vesicles in pumpkin seeds. Again, the heterologous expression of the storage protein caused the formation of novel structures in vegetative tissues of *Arabidopsis* that were similar to PAC vesicles. The vesicles did not apparently fuse with the vacuole, and therefore, while the machinery for the formation of the vesicles is likely to be present in all cells and all plant species, the machinery for uptake into the vacuole may only be present in seeds. This could be due to many vegetative cells containing only a lytic vacuole, rather than a protein storage vacuole. Alternatively, the PAC-like vesicles could be delivered to the vacuole and their contents rapidly degraded due to the high activity of lytic enzymes in the lytic vacuole.

III. TRANSPORT TO THE VACUOLE DURING SEED GERMINATION

The large quantities of storage proteins that are transported to the vacuole during seed development must be broken down by proteases during germination to support the seedling until photosynthesis takes over. There has been some debate over whether the proteases for this breakdown are synthesized *de novo* after

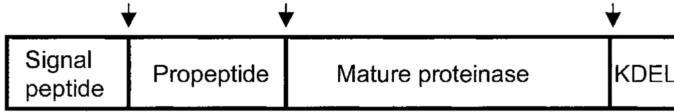

Fig. 5. Structure of KDEL-tailed proteinase. Proteinases from mung bean, castor bean and many other plant species contain an N-terminal signal peptide for cotranslational transport into the ER, an N-terminal propeptide, and a KDEL ER retention signal at the C-terminus. Arrows indicate the sites where cleavage produces the mature, active proteinase.

germination, or whether they are synthesized during seed development and either stored in an inactive form or sequestered away from their substrates until their activity is required. The reality is probably a combination of these possibilities, dependent on the plant species and on the protease under study. Many proteases are likely to be transported to the vacuole by the classical Golgi-dependent pathways (section II). However, some alternative transport pathways have recently been described for the bulk transport of proteases during seed germination.

A. PROTEASE-CONTAINING VESICLES BUD FROM THE ER

1. *Role in Storage Protein Mobilization*

Vigna mungo seeds have been used as a system to study the rapid mobilization of storage proteins after germination. A cysteine protease (called SH-EP) was shown to be capable of breaking down seed globulin (Okamoto and Minamikawa, 1998) and was proposed to be a major factor responsible for its degradation upon seed germination. SH-EP is synthesized as a preproprotein at the ER and cotranslationally processed to the proform. The propeptide is later removed to produce the enzymatically active mature form, either autocatalytically or by an asparaginyl endopeptidase that was also identified from *Vigna mungo* seeds (Fig. 5; Okamoto and Minamikawa, 1999).

One unusual feature of SH-EP is the presence of a KDEL sequence at the C-terminus of the protein (Okamoto *et al.*, 1999). KDEL and related sequences are found in ER-resident proteins and function as signals for the retrieval of escaped proteins back from the Golgi complex. One function of the SH-EP KDEL signal may be to prevent the forward transport of the protein out of the ER and through the Golgi complex. SH-EP therefore accumulates to high levels in the ER as the relatively inactive proform, until germination of the seeds.

Seed storage protein breakdown within cotyledon cells of germinated *Vigna mungo* seeds was seen to occur very rapidly (Toyooka *et al.*, 2000), and the potential role of SH-EP in this process was analyzed. Immunogold electron microscopy using SH-EP antibodies revealed the accumulation of SH-EP in large (200–500 nm), single-membrane vesicles that were distinct from the protein storage vacuole. These vesicles were designated KVs, for ‘KDEL-tailed

cysteine proteinase-accumulating vesicles'. These vesicles could not be seen in cotyledon cells of maturing seeds, only in seeds after germination, and therefore are probably formed *de novo* upon germination (Toyooka *et al.*, 2000). In addition to the KVs, SH-EP labeling was seen in swollen regions of the ER, and KVs were often seen close to or associated with the ER, suggesting that the KVs may bud directly from the ER. In contrast, no SH-EP labeling could be seen over the Golgi apparatus. Antibodies against complex glycans, added to proteins during passage through the Golgi apparatus, stained the Golgi but not the KVs. Together, these data indicate that the KVs, and the SH-EP that they contain, are transported via a Golgi-independent pathway to their final destination of the protein storage vacuole.

Electron micrographs were obtained that appeared to show the fusion of KVs with the protein storage vacuole. After fusion of the two membranes, SH-EP would presumably be released into the vacuole, where breakdown of storage proteins could proceed. Antibodies against the mature portion of SH-EP labeled both the KVs and the protein storage vacuoles, whereas antibodies specific to the proform of SH-EP labeled predominantly the KVs and not the protein storage vacuoles (Toyooka *et al.*, 2000). It is therefore likely that the proteinase is transported in KVs as the proenzyme and activated by removal of the propeptide during or after fusion of the KV with the storage vacuole. The potential SH-EP processing enzyme (asparaginyl endopeptidase) is transported to the vacuole through the Golgi apparatus, not via KVs, providing one possible means of preventing premature activation of SH-EP in the KVs. In addition, a cysteine protease capable of removing the SH-EP C-terminal propeptide, which contains the KDEL signal, was purified and localized to both the ER and the protein storage vacuole (Okamoto *et al.*, 2001).

A putative receptor for SH-EP (named VmVSR) was isolated from cotyledons of germinating seedlings by affinity chromatography using immobilized proSH-EP (Tsuru-Furuno *et al.*, 2001). The receptor protein showed a high sequence similarity to the vacuolar sorting receptor family (Fig. 4) and was able to bind to the N-terminal propeptide of SH-EP. A second, closely related protein, termed VmVSR homolog, was also identified from *Vigna mungo*, and could also bind to the SH-EP prosequence. However, the expression patterns of the two potential receptors, and subcellular fractionation experiments, indicated that only VmVSR is expressed in the germinating seeds at the appropriate time and cofractionates with SH-EP. VmVSR itself is therefore likely to be the authentic SH-EP receptor, and VmVSR homolog is probably involved in other transport processes, such as Golgi-mediated vacuolar protein targeting. This may be analogous to the case with PV72 (see section II.B), which is found in PAC vesicles and may also play a role in Golgi-independent vacuolar trafficking. A common theme in vacuolar protein transport in seeds may be the involvement of specific members of the vacuolar sorting receptor family in Golgi-dependent and Golgi-independent trafficking pathways. At least in the case of *Vigna mungo*, specificity could be attained by differences in the timing of expression and the

subcellular localization of individual members of the family, rather than binding specificity for different propeptide signals.

2. Role in Programmed Cell Death

While a role for SH-EP in mobilization of seed storage proteins from the protein storage vacuole during germination has been proposed, a different function has been suggested for a closely related protease in castor bean (*Ricinus communis*). In this case, a cysteine endopeptidase (CysEP) was isolated fortuitously by its ability to process a glyoxysomal protein (Gietl *et al.*, 1997). However, it appears that this is not its *in vivo* role, due to the physical separation of enzyme and potential substrate; CysEP is not detectable in glyoxysomes. Instead, it is found in endosperm cells of germinating castor bean in organelles termed ricinosomes, which appear to be equivalent to the KVs described in *Vigna mungo*.

The structures of SH-EP from mung bean and CysEP from castor bean are very similar (Fig. 5). Like SH-EP, CysEP is synthesized as a preproprotein, with an N-terminal ER targeting signal that is removed cotranslationally as the protein is translocated into the ER lumen. ProCysEP is enzymatically active, although with a much lower activity than the mature protein. The sequence reveals the presence of a C-terminal KDEL motif for ER retention.

The organelles containing CysEP (ricinosomes) were seen by electron microscopy to be large organelles of approximately 900 nm in diameter, slightly larger than that described for *Vigna mungo* KVs. The ricinosomes were surrounded by a single membrane that sometimes had associated ribosomes, suggesting that, like KVs, ricinosomes are also derived from the ER. Purified ricinosomes also contained small amounts of the ER chaperones BiP and PDI (protein disulphide isomerase), in addition to CysEP, supporting an ER origin. The CysEP in isolated ricinosomes still contained the KDEL motif and the N-terminal propeptide, although acidification of the ricinosomes *in vitro* led to cleavage to the mature form of the protease (Schmid *et al.*, 2001), presumably autocatalytically. It appears that, like SH-EP, CysEP initially accumulates in the ER as a proprotein by virtue of its KDEL sequence. Expansion of the ER lumen follows, and ricinosomes eventually bud off as large vesicles studded with ribosomes.

Unlike KVs, however, ricinosomes do not appear to fuse with the vacuole, but rather remain in the cytoplasm until after storage protein mobilization is complete. Schmid *et al.* (1999) proposed an alternative explanation for the occurrence and function of ricinosomes. They suggest that the ricinosomes containing proCysEP remain intact until the final stages of senescence of the endosperm cells. At this point the ricinosomes are thought to disintegrate, releasing the protease into the cytoplasm. Acidification of the cytoplasm upon loss of integrity of the vacuolar membrane, a late stage in the senescence process, would lead to processing of proCysEP to the mature, highly active form. It is proposed that the protease is then involved in the degradation of the remaining cell contents and thus in nutrient scavenging for the germinating seedling, before the final death and collapse of the endosperm cells.

Additional support for a role of CysEP in programmed cell death during senescence was obtained by the study of KDEL-containing proteases in other tissues and species. In castor bean, ricinosomes containing CysEP were also identified in developing seeds, in a layer of collapsing cells of the seed coat (Schmid *et al.*, 1999). Antibodies against CysEP labeled ricinosome-like organelles in the petals of *Hemerocallis* (daylily) flowers undergoing senescence (Schmid *et al.*, 1999). The expression patterns of three different *Arabidopsis* KDEL-containing proteases with homology to CysEP were analyzed using transgenic *Arabidopsis* plants containing promoter–GUS fusions. Each gene gave a unique expression pattern, in each case in tissues that were undergoing senescence (Gietl and Schmid, 2001). While it is not known whether the proteases are present in ricinosome-like organelles and involved in programmed cell death, together, the above results implicate the KDEL-containing family of proteases in nutrient mobilization during cell senescence.

3. *Role in Stress Responses*

Another kind of ER-derived organelle has recently been described in *Arabidopsis* epidermal cells of young seedlings (Hayashi *et al.*, 2001) that may also be involved in cell death. The organelles were originally observed in cotyledons of transgenic *Arabidopsis* plants containing ER-targeted green fluorescent protein (GFP; Gunning, 1998). In addition to the characteristic network of ER fluorescence, large organelles of approximately 5 μm in length could be seen to move rapidly in the cytoplasm. Electron microscopy and labeling with anti-GFP antibodies showed that the organelles form by dilation of the ER cisternae, and were named ER bodies. The ER bodies were studded with ribosomes, similar to KVs and ricinosomes, but typically were considerably larger than either, and the authors propose that the *Arabidopsis* ER bodies are a novel kind of ER-derived organelle with a distinct function (Hayashi *et al.*, 2001).

A clue as to the function of the ER bodies came from the observation that two different vacuolar cysteine proteases (RD21 and γVPE) accumulated within them (Hayashi *et al.*, 2001). Unlike SH-EP and CysEP, these proteases do not contain a KDEL ER retention signal, so the mechanism by which they are retained in the ER-derived bodies, rather than being transported on to the vacuole, is unclear. Differential centrifugation experiments indicated that the precursor form of RD21 accumulated in the ER bodies, whereas the mature form was found in vacuoles, implicating the ER bodies as a transport intermediate between the ER and vacuoles.

Both RD21 and γVPE proteases are stress-inducible under various conditions (Koizumi *et al.*, 1993; Kinoshita *et al.*, 1999, and see Fig. 7). Salt stress of *Arabidopsis* seedlings led to the fusion of GFP-tagged ER bodies with each other in the epidermal cells, visualized by fluorescence microscopy (Hayashi *et al.*, 2001). Eventually, the ER bodies fused with vacuoles, followed by death of the cells, seen as loss of membrane impermeability. Electron microscopy studies allowed the elucidation of the pathway in more detail. First, the

membranes of the ER body and vacuole became closely apposed, with loss of ribosomes from the ER body membrane at the contact site. The ER body then began to be engulfed by the vacuole, and the membranes in between the two organelles were no longer visible. Finally, the contents of the ER body were released into the vacuole.

The function of ER bodies therefore appears to be a regulated pathway of delivery of proteins to the vacuole. It is hypothesized that, in *Arabidopsis* seedlings, the ER bodies store proteinase precursors in preparation for an encounter with stress conditions. When challenged with an environmental stress, the ER bodies mediate a mass delivery of the precursors to the vacuole, where they are processed to the mature form and may function in nutrient scavenging for the seedling as the epidermal cells die.

From the above, it is clear that many plant species produce large, ER-derived vesicles containing proteinase precursors (Chrispeels and Herman, 2000). While these have many features in common, a number of differences have also been described, such as the presence of KDEL signals at the C-termini of the proteases, and in particular whether the vesicles eventually fuse with the vacuole, as for KVs of mung bean and ER bodies of *Arabidopsis*, or whether the contents are released directly into the cytoplasm, as for ricinosomes in castor bean. Different functions have also been ascribed to the vesicles, from seed storage protein degradation to programmed cell death. Future research should elucidate whether these are entirely separate pathways or whether they simply represent variations of the same mechanism that is adapted to different roles in different species.

B. AUTOPHAGY DURING SEED GERMINATION

Upon seed germination in certain species, in addition to the degradation of storage proteins by vacuolar proteases, other cell components are taken up into vacuoles and degraded (Fig. 6). This is assumed to be an additional method for recycling unneeded organelles, as after mobilization of storage proteins, the storage tissues may be considered as senescing organs (see section III.A.2). At early stages of seed germination in mung bean, in addition to containing storage proteins, vesicles containing cytoplasmic contents could be seen inside protein bodies (Van der Wilden *et al.*, 1980; Herman *et al.*, 1981). At later stages, the vesicles appeared empty, suggesting that the contents had been degraded by vacuolar hydrolases. The pathway for uptake of the vesicles was indicated by an ultrastructural study in cotyledon cells (Toyooka *et al.*, 2001).

Autophagosomes consisting of a double membrane surrounding cytoplasmic contents could be seen in the cytoplasm. Autophagic bodies containing cytoplasmic organelles could also be observed inside the vacuole in these cells, and an autophagosome was observed that was apparently in the process of fusing with the vacuole. This process occurred even under conditions where starch granules were not taken up into the vacuole (see section VI), indicating that two different mechanisms of autophagic vacuolar uptake operate in the germinating seedlings, one for the uptake of cytoplasmic organelles and another for starch grains.

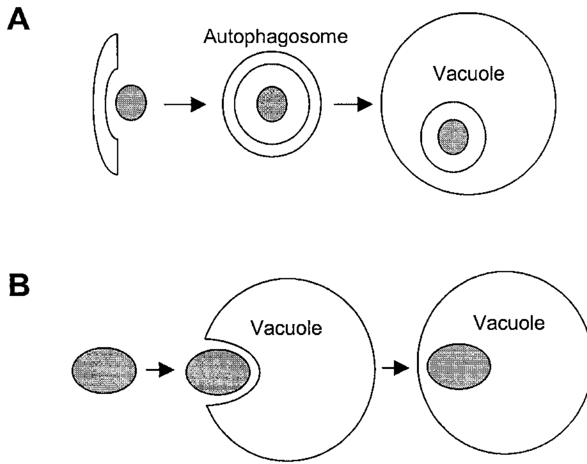

Fig. 6. Pathways for vacuolar autophagy. (A) Macroautophagy. Cytoplasmic organelles and other constituents are surrounded by a membrane structure, possibly derived from the endoplasmic reticulum, that seals to form a double-membraned autophagosome. The outer membrane of the autophagosome fuses with the vacuole membrane, releasing the inner membrane containing the cytoplasm into the vacuole, where it is degraded. (B) Microautophagy. Organelles associate with, and are engulfed by, the vacuole. The vacuolar membrane seals around the engulfed component, which is degraded by vacuolar hydrolases.

IV. STRESS-INDUCED VACUOLAR AUTOPHAGY

A. VACUOLAR PROTEASE ACTIVITY INCREASES DURING STRESS

In plants, variations in many different environmental factors can lead to a carbohydrate deficit in some cells. For example, constant changes that occur in light and temperature can limit photosynthetic efficiency and therefore the supply of carbohydrate. When plants are exposed to carbon starvation stresses, they begin a process of nutrient mobilization to enable essential functions for life to continue at the expense of those that are non-essential, at least in the short term.

Vacuolar proteolysis plays a critical role in protein recycling under such stress conditions. When plants are under non-stressed conditions, the vacuole contains significant protease activity, that may, along with the proteasome system (Vierstra and Callis, 1999), play a role in general cell homeostasis and removal of damaged and unneeded proteins. When subjected to nutrient stress, in particular carbon starvation, cellular protease activity increases dramatically. James *et al.* (1993) studied the changes in protease activity in excised maize root tips during glucose starvation over several days. Serine and cysteine protease activity increased during this time from low levels in non-starved roots, and this increase was correlated with a degradation of proteins

in the root tips and a transient increase in free amino acids, suggesting that the protease activity is responsible for recycling of the amino acids for cell survival. The proteases that were induced during these conditions had acidic pH optima, implicating the vacuole as the site of protein degradation, rather than the cytosol. The protease induction was inhibited in root tips supplied with exogenous glucose, showing that the increase is not simply a consequence of excision of the root tips.

This study was later extended to whole maize plants, when exposed to periods of extended darkness (Brouquisse *et al.*, 1998). Endopeptidase activity in roots and root tips increased after two days in darkness. This activity was attributed to the induction of a predominant vacuolar serine endopeptidase (RSIP), both in the whole plants and in excised root tips (James *et al.*, 1996).

In addition to increases in protease activity, the expression of genes encoding several vacuolar enzymes was upregulated upon sucrose starvation in *Arabidopsis* suspension cells (Fig. 7). These include vacuolar invertase (Tymowska-Lalanne and Kreis, 1998), vacuolar processing enzyme (Kinoshita *et al.*, 1999) and aleurain (Griffiths *et al.*, 1997), each of which is known to be stress induced in whole plants.

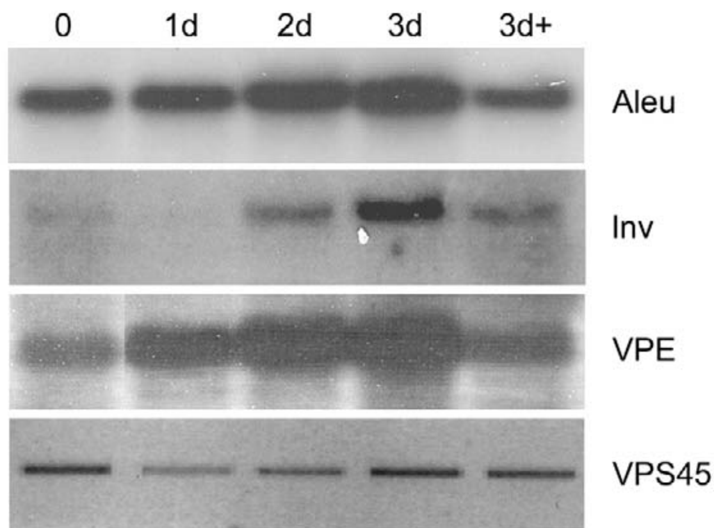

Fig. 7. Changes in RNA levels on sucrose starvation of suspension cells. *Arabidopsis* suspension cultures were grown without sucrose for the indicated times (zero to three days) or in the presence of sucrose for three days (3d+) and RNA was extracted. RNA levels were analyzed by northern blotting using radiolabeled probes corresponding to aleurain (Aleu), vacuolar invertase (Inv) or vacuolar processing enzyme-γ (VPE), or by RT–PCR (reverse transcription–polymerase chain reaction) using primers specific to the *VPS45* gene as a control (VPS45).

B. MORPHOLOGICAL CHANGES IN PLANT CELLS DURING STRESS-INDUCED AUTOPHAGY

If vacuolar proteases are involved in the breakdown of cellular organelles during nutrient stress, mechanisms must exist for the uptake of cytoplasm by the vacuole under these conditions. The morphological effect of carbon deprivation on plant cells has been studied most extensively in suspension cultures, as this system allows coordinate induction of stress responses in a relatively homogeneous population of cells. Cell lines derived from several different plant species have been shown to undergo similar morphological changes at the subcellular level upon sucrose limitation. Chen *et al.* (1994) used rice suspension cells as a model for studying the control by sugar of the breakdown of starch in cereals. After transfer of the cells to a sucrose-free medium, their ultrastructure was examined by electron microscopy. Extreme changes in subcellular morphology were observed upon starvation. Cells grown in sucrose contained many starch grains and organelles, including small vacuoles. Upon sucrose starvation, the size of the vacuole increased dramatically, taking up the majority of the cell volume. Most of the other organelles disappeared, and the remainder were pressed to the outside of the cells, adjacent to the plasma membrane. A similar effect on subcellular structure can be seen in other species of plant cell, including suspension-cultured cells of *Arabidopsis thaliana* (Fig. 8).

Similar morphological changes were seen in tobacco suspension-cultured cells after a day of sucrose starvation (Moriyasu and Ohsumi, 1996), and were suggested to be due to an induction of vacuolar autophagy (Fig. 6). In the presence of cysteine protease inhibitors, but not inhibitors of other classes of proteases, spherical bodies accumulated. These structures were acidic and contained acid phosphatase activity, and may correspond to autophagosomes seen in yeast

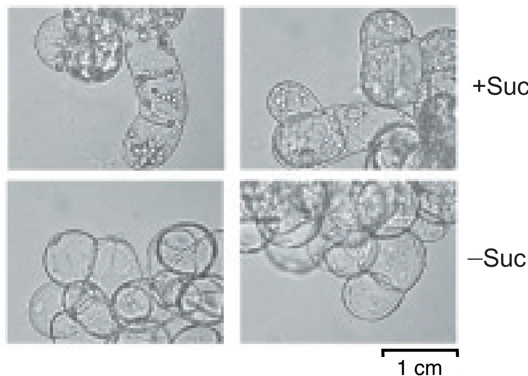

Fig. 8. Morphology of *Arabidopsis* suspension cells after starvation. Cells were grown for three days in the presence (upper panels) or absence (lower panels) of sucrose. Cells grown without sucrose showed dramatic morphological changes, with enlargement of the vacuole and disappearance of other organelles.

and mammalian cells. A more detailed description of autophagy using electron microscopy in sycamore suspension cells, revealed the pathway for breakdown of cytoplasm during sucrose starvation (Aubert *et al.*, 1996). Autophagosomes were formed as double-membrane vesicles in the cytoplasm, and engulfed by the vacuole, by a mechanism that is unclear, to be released as single-membrane vesicles inside the vacuole lumen. The presence of membrane remnants inside the vacuole indicated a breakdown of the vesicles by vacuolar enzymes. The total number of mitochondria (and presumably other organelles) decreased during sucrose starvation (Journet *et al.*, 1986). It is thus hypothesized that membranes from an unknown source wrap around portions of cytoplasm, including organelles such as mitochondria, but excluding the nucleus, eventually completely enclosing them within a double-membrane autophagosome. This autophagosome travels to the vacuole where the outer membrane of the autophagosome fuses with the tonoplast, releasing the inner vesicle with its cytoplasmic contents into the vacuolar interior. This vesicle and its contents are then broken down by the action of vacuolar hydrolases to release the constituents for recycling to maintain essential cell functions.

The control of autophagy induction in plant cells during starvation conditions has been studied. Chen *et al.* (1994) suggested that vacuolar autophagy in rice cells is regulated by the sugar level in the medium, as removal of sucrose leads to the induction of autophagy. Aubert *et al.* (1996) addressed this by growth of suspension cells on different carbon sources, and determined that autophagy did not occur in the presence of glycerol or pyruvate, even after almost complete depletion of sucrose in the cells. They therefore proposed that, rather than the concentration of sugar and sugar phosphates, the reduction in the supply of respiratory substrates to mitochondria may be the trigger for autophagy in these cells.

C. MECHANISM OF AUTOPHAGY IN YEAST AND MAMMALIAN CELLS

While the process of vacuolar autophagy in plants has been described morphologically, little information is available about the proteins that may be required for this process. In contrast, genetic screens in yeast have allowed the identification of genes that are required for various steps of the autophagy process in response to nutrient deprivation. Mutants defective in autophagy are extremely sensitive to nutrient stress and rapidly lose viability when transferred to these starvation conditions. This starvation-induced autophagic pathway also overlaps extensively with a constitutive pathway for the transport of a subset of proteins to the vacuole, the Cvt (cytoplasm-to-vacuole transport) pathway (Klionsky and Ohsumi, 1999).

Screens for yeast mutants impaired in this process have identified a novel protein conjugation system that appears to be central to the formation of autophagosomes and that is related to the ubiquitination system for protein degradation (Mizushima *et al.*, 1998a; Ichimura *et al.*, 2000). The conjugation system is conserved in mammalian cells, underlining its importance to the basic

autophagy process (Mizushima *et al.*, 1998b). It involves the covalent attachment of Apg5p to Apg12p, and two enzymes are essential for this conjugation process. The first is Apg7p, which shows sequence similarity to E1 ubiquitin-activating enzymes, and the second is Apg10p, which appears to function as a conjugating enzyme. Each of these proteins is required for vacuolar autophagy; however, their precise role in the autophagy process remains to be determined. Apg7p is also needed as the activating enzyme for a different conjugation process, that of Aut7p, another protein required for autophagy. Aut7p, rather than being conjugated to another protein, is attached to phosphatidylethanolamine, by the action of the conjugating enzyme Aut1p (Ichimura *et al.*, 2000).

Other genes involved in yeast autophagy include those encoding proteins that function in autophagosome formation, fusion with the vacuole, and breakdown inside the vacuole. A few genes have been identified that may encode components of the autophagy signaling pathway; Apg13p and Apg1p may regulate the formation of autophagic vesicles in response to changes in nutrient availability (Scott *et al.*, 2000). Finally, Tor is a phosphatidylinositol kinase-related protein that is conserved in yeast and mammalian cells and is a regulator of many responses to nutrient availability, including autophagy as well as gene expression and growth (Noda and Ohsumi, 1998; Rohde *et al.*, 2001).

D. *ARABIDOPSIS* CONTAINS HOMOLOGS OF YEAST AUTOPHAGY GENES

Searches of the *Arabidopsis thaliana* genome sequence database have revealed that many of the genes required for autophagy in yeast have sequence homologs in *Arabidopsis*. It has been observed previously that, for a single vesicle trafficking gene in yeast, a small gene family with several closely related members often exists in *Arabidopsis* (Bassham and Raikhel, 2000). This does not appear, at least in many cases, to reflect functional redundancy, but rather a specialization of functions in the highly complex plant endomembrane system (e.g. Zheng *et al.*, 1999; Sanderfoot *et al.*, 2001). This also seems to be the case for genes involved in autophagy. For example, Aut7p is a yeast protein that is upregulated during autophagy and may be required for autophagosome expansion (Abeliovich *et al.*, 2000). As mentioned above, it undergoes a unique lipidation event that is also required for autophagy. Database searching reveals homologs in many species, including several in mammals that are known to function in aspects of vesicle trafficking. Eight *Arabidopsis* genes that are closely related to yeast *AUT7* could be identified (Fig. 9) and are good candidates for genes playing a role in autophagy and other related trafficking pathways. The *Arabidopsis* genes cluster into three groups, and it will be interesting to determine whether this has functional relevance. For example, proteins from each group could potentially reside on a different subcellular organelle, or function in a distinct vesicle transport pathway.

Predicted proteins with sequence similarity to other yeast autophagy proteins could also be identified in *Arabidopsis* (Table I). In some cases the sequence

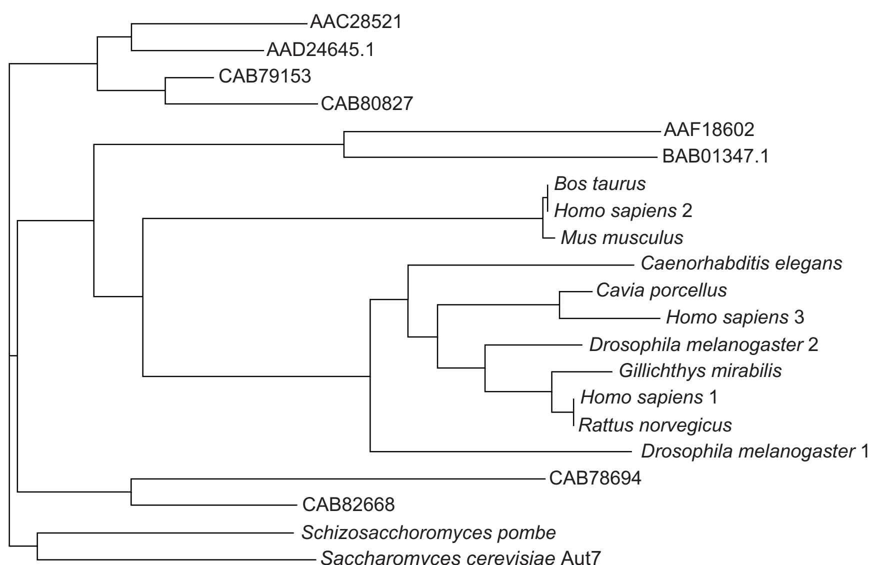

Fig. 9. Comparison of Aut7p-like proteins. A phylogenetic tree of Aut7p-like proteins from various species was generated using the Vector NTI suite of sequence analysis programs. Accession numbers refer to sequences from *Arabidopsis*. Protein IDs for other species are as follows: *B. taurus*, 4433387; *C. elegans*, 17535125; *C. porcellus*, 13507165; *D. melanogaster* 1, 7300298; *D. melanogaster* 2, 7291184; *G. mirabilis*, 10121677; *H. sapiens* 1, 6005764; *H. sapiens* 2, 6005768; *H. sapiens* 3, 14211879; *M. musculus*, 13386158; *R. norvegicus*, 5712778; *S. cerevisiae*, 6319393; *S. pombe*, 7492425.

TABLE I

Identification of Arabidopsis *homologs of yeast autophagy genes. The amino acid sequence of yeast autophagy proteins was used to search predicted proteins from the* Arabidopsis *genome sequence using the BLAST algorithm. Sequence alignments were performed using the Vector NTI suite of programs*

Yeast autophagy protein	*Arabidopsis* gene accession number	% amino acid identity
Apg5	At5g17290	16
Apg6	At3g61710	19
Apg7	At5g45900	36
Apg9	At2g31260	16
Aut2	At2g44140	18
Aut2	At3g59950	19
Aut10	At2g40810	24
Aut10	At5g05150	18
Aut10	At3g62770	19
Aut10	At3g56440	20

similarity is strong; for example, yeast Apg7 protein shares about 36% amino acid sequence identity with its putative *Arabidopsis* homolog, and *Arabidopsis* Aut7p-like proteins (Fig. 9) share up to 70% identity with yeast Aut7p. Others, such as Apg5p, show much lower, but still significant, sequence identities. Interestingly, there are some yeast autophagy proteins (e.g. Apg14p and Aut4p) for which no related protein can be found in *Arabidopsis*, based on the completed genome sequence. One possibility is that the autophagy mechanism in plants is different from that in yeast, such that these proteins are not required. Alternatively, other *Arabidopsis* proteins, unrelated in sequence to the yeast proteins, may nevertheless fulfill the same function.

V. AUTOPHAGY AND VACUOLE FORMATION

In meristematic cells, such as those of the root tip, and in developing pea cotyledon cells (Hoh *et al.*, 1995), vacuoles form *de novo* from tubules that may be derived from the ER or the *trans*-Golgi network. While the molecular details of this process remain to be elucidated, morphologically it resembles autophagosome formation (Marty, 1999; Robinson *et al.*, 1998). Vesicles and tubules fuse to form a cage-like structure surrounding a portion of cytoplasm. Eventually, a complete membrane is formed that becomes the tonoplast, and the cytoplasmic components and excess membrane are degraded inside the newly formed vacuole. These new, small vacuoles can then fuse to create a large central vacuole as the cell matures and differentiates. The membrane source and mechanism for the formation of autophagosomes under stress conditions may thus be related to vacuole formation in meristems.

VI. DEGRADATION OF STARCH IN THE VACUOLE

In addition to the degradation of proteins in the vacuole after autophagy, the breakdown of starch grains has also been observed, both during nutrient depletion and seed germination. Alpha-amylase is a key enzyme in the starch degradation process, and is known to be regulated by sucrose (Yu *et al.*, 1992). Chen *et al.* (1994) identified a group of α-amylases that accumulated in rice suspension cells after transfer to sucrose-free growth medium for 1 day, and then were degraded when the cells were transferred back to sucrose-containing medium. The amount of starch in the starved cells decreased rapidly, presumably due to breakdown by these α-amylases. A time course of starvation demonstrated that morphological changes typical of the induction of autophagy were identifiable after a few hours without sucrose, and at the same time, starch granules were visible inside the vacuole. Immunogold labeling demonstrated that some of the α-amylase was present inside the vacuole, and the vacuole therefore may be the site of starch degradation during sucrose starvation. The authors postulate that vacuolar autophagy is responsible for the large increase in vacuolar volume and

concomitant decrease in cytoplasmic content, including the uptake of amyloplasts by the vacuole.

These observations were extended to the degradation of starch granules in cotyledons of germinating *Vigna mungo* seedlings (Toyooka *et al.*, 2001). In this system, α-amylase was found in both the protein storage vacuole and in the lytic vacuole, which may be derived from the protein storage vacuole after degradation of seed storage proteins. No α-amylase could be detected associated with starch granules, indicating that starch granules may be degraded after uptake by the vacuole. A sequence of events in the breakdown of starch granules was deduced by comparison with the degradation of storage proteins in cotyledons of germinated seeds, observed as a decrease in electron density of the protein storage vacuoles. After the initiation of storage protein degradation, membrane structures surrounded the starch granules, and an area of low electron density was seen around the starch granules, which increased in size as the storage protein reserves were depleted. Vesicles of a similar electron density, with acidic contents, apparently formed *de novo* after seed germination, were seen at this time in the cytoplasm, and may be the source of the structures around the starch granules. After conversion of the protein storage vacuole to a lytic vacuole, the membrane around the starch granule was seen to fuse with the tonoplast, delivering the starch granule into the vacuole. The starch granule was then broken down inside the lytic vacuole by α-amylase.

VII. VACUOLAR AUTOPHAGY AND LEAF SENESCENCE

The process of leaf senescence is a type of programmed cell death requiring the ordered breakdown of individual cell components for recycling to the rest of the plant. There has been much discussion over the role of the vacuole in the various stages of leaf cell senescence (see Matile, 1997 for a review). In particular, whether cellular organelles and cytoplasmic constituents are taken up into the vacuole (e.g. by autophagy) for degradation has been the subject of some debate. As in stressed cells, vacuolar hydrolase activity increases significantly in senescing leaves, and therefore presumably has a function in macromolecular degradation in the leaf cells. Two main possibilities can be envisaged for this function. The vacuole could be an autophagic compartment that engulfs portions of cytoplasm, including whole organelles, and degrades them to provide nutrients for export to other tissues (Fig. 6). This would occur while tonoplast membranes still retained their semipermeability, before death of the cell. Alternatively, the hydrolases could accumulate in preparation for their ultimate release into the cytosol after loss of vacuolar integrity. Their function would thus be to degrade the remaining cytoplasmic components during the final stages of death of the cell. Again, the products of degradation would ultimately be transferred to the non-senescing plant tissues for re-use.

As the major leaf protein is RuBisCO (ribulose 1,5-bisphosphate carboxylase/oxygenase), the breakdown of chloroplasts and chloroplast proteins is clearly

significant with respect to recycling of amino acids from senescing tissue. Several vacuolar proteases have been described that have the capacity to degrade RuBisCO (e.g. see Lin and Wittenbach, 1981; Miller and Huffaker, 1982). However, many vacuolar proteases are relatively non-specific, and determination of their function *in vivo* in the breakdown of specific proteins is difficult. Protease activities capable of breaking down RuBisCO have also been described within the chloroplast (e.g. see Ragster and Chrispeels, 1981; Bushnell *et al.*, 1993). It is possible that both plastid and vacuolar proteolytic systems are involved in chloroplast protein degradation, and the extent to which each is used depends on factors such as the plant species and environmental conditions.

Morphological studies have provided some support for the uptake of a population of chloroplasts into the vacuole during senescence. Wittenbach *et al.* (1982) reported that the number of chloroplasts in senescing wheat leaf cells decreased as senescence progressed. At the same time, some chloroplasts could be seen to have moved away from the plasma membrane, and possibly to have been taken up into the vacuole by an autophagic mechanism. Recently, an extension of these observations by Minamikawa *et al.* (2001) provided additional evidence that chloroplasts are associated with the vacuole during senescence of detached French bean leaves. Using electron microscopy, chloroplasts could be seen within the vacuole, and at later time points these chloroplasts were partially degraded, and membrane fragments, potentially derived from the chloroplasts, were found inside the vacuole. Immunogold labeling revealed that a vacuolar cysteine protease was associated with the chloroplasts undergoing degradation, and it was postulated that this protease is involved in the breakdown of RuBisCo large subunit.

One limitation of most of these studies on leaf senescence is that the experiments are performed on leaves detached from the plants, as a convenient but somewhat artificial experimental system. It will be interesting to see whether the results hold for leaves senescing naturally on the plant.

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NOTE ADDED IN PROOF

Recent evidence corroborates the hypothesis that there is a link between autophagy and senescence in plants. Two Arabidopsis mutants have been identified with defects in genes showing sequence similarity to yeast autophagy genes. Both *atapg9* (Hanaoka *et al*., 2002) and *atapg7* (Doelling *et al*., 2002) mutants display an increased sensitivity to nutrient deficiency and early senescence phenotype.

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Phosphoenolpyruvate Carboxykinase: Structure, Function and Regulation

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Abbreviations used are the following: GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GS, glutamine synthetase; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PEPC, phophoenolpyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; RuBisCO, ribulose-1,5-bisphosphate carboxylase.

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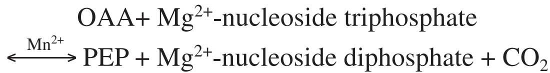
ABSTRACT

The aim of this article is to outline our understanding of the enzyme phosphoenolpyruvate carboxykinase (PEPCK). Although emphasis is placed on the enzyme derived from flowering plants, other organisms are also considered, because comparative studies provide invaluable information. The following points are considered in detail. Firstly, the possibility that PEPCK in all organisms arose from a common ancestor, and that the extension of about 12 kDa at the N-terminus of PEPCK–ATP from flowering plants, which is not possessed by PEPCK–ATP from other organisms, is homologous to the N-terminal region of PEPCK–GTP. Secondly, the regulation of PEPCK activity in flowering plants by reversible protein phosphorylation is described. Phosphorylation of the N-terminal extension possessed by PEPCK from flowering plants reduces its catalytic velocity several-fold at physiological concentrations of oxaloacetate. How this is likely to contribute to regulation of PEPCK *in vivo* is described. Thirdly, it is proposed that in flowering plants PEPCK plays a widespread role as a component of a mechanism that counteracts intracellular acidification. The proposed role of PEPCK in this mechanism is similar to that in the kidney during acidosis.

I. INTRODUCTION

The aim of this article is to outline our understanding of the enzyme phosphoenolpyruvate carboxykinase (PEPCK). Although emphasis is placed on the enzyme from flowering plants other organisms are considered because comparative studies provide invaluable information. PEPCK catalyses the reversible reaction:



$$\text{OAA} + \text{Mg}^{2+}\text{-nucleoside triphosphate} \xleftrightarrow{\text{Mn}^{2+}} \text{PEP} + \text{Mg}^{2+}\text{-nucleoside diphosphate} + \text{CO}_2$$

where OAA = oxaloacetate; PEP = phosphoenolpyruvate

The following points are considered in detail. Firstly, the possibility that PEPCK in all organisms arose from a common ancestor, and that the extension of about 12 kDa at the N-terminus of PEPCK–ATP from flowering plants, which is not possessed by PEPCK–ATP from other organisms, is homologous to the N-terminal region of PEPCK–GTP. Secondly, the regulation of PEPCK activity in flowering plants by reversible protein phosphorylation is described. Phosphorylation of the N-terminal extension possessed by PEPCK from flowering plants reduces its catalytic velocity several-fold at physiological concentrations of oxaloacetate. The way in which this is likely to contribute to regulation of PEPCK *in vivo* is described. Thirdly, it is proposed that in flowering plants PEPCK plays a widespread role as a component of a mechanism that counteracts intracellular acidification. The proposed role of PEPCK in this mechanism is similar to that in the kidney during acidosis. The reader is referred to the following for detailed accounts: the early work on PEPCK from all organisms (Utter and Kolenbrander, 1972), the discovery of PEPCK (Kurahashi, 1985), studies on PEPCK from vertebrates (Hanson and Patel, 1994), regulation of the abundance of PEPCK in vertebrates (Hanson and Reshef, 1997), and the structure and mechanism of PEPCK (Matte *et al.*, 1997).

II. PEPCK FROM FLOWERING PLANTS: A HISTORICAL PERSPECTIVE

Work on PEPCK can be traced back to studies whose aim was to establish the enzymatic basis of light-independent fixation of CO_2 into dicarboxylic acids. For example, leaves of certain succulent plants accumulate organic acids during the night, which are lost during the day (see Thomas, 1949), similarly, animal tissues and bacteria fix CO_2. Wood and Werkman (1936) proposed a reaction that involved the direct carboxylation of pyruvate to form oxaloacetate in bacteria. In the early 1940s Hastings and his co-workers used carbon isotopes to show that, in liver, glycogen synthesis from pyruvate involved CO_2 fixation and a symmetrical dicarboxylic acid intermediate (see Utter and Kolenbrander, 1972).

In 1953 Merton Utter and Kiyoshi Kurahashi discovered phosphoenolpyruvate carboxykinase (GTP) in chicken liver, which initially was thought be an answer to how CO_2 was fixed in that organ. However, subsequent work showed that this was not the case (see Utter and Kolenbrander, 1972). In 1963 Utter and Keech discovered pyruvate carboxylase, which is responsible for the fixation of CO_2 into dicarboxylic acids in most vertebrate tissues. Pyruvate carboxylase has not been found in flowering plants. Bandurski (1955) discovered phosphoenolpyruvate carboxylase (PEPC) in spinach leaves; subsequent studies showed that PEPC was widely distributed in flowering plants (see Tchen and Vennesland, 1955; Mazelis and Vennesland, 1957). This enzyme is responsible for light-independent CO_2 fixation in most tissues of flowering plants, but is not present in many organisms, including animals. Tchen and Vennesland (1955) provided evidence that wheat-germ extracts caused the enzymatic formation of OAA from PEP and CO_2, by two different reactions. One of these was reversible and required ADP as a phosphate acceptor. These were characteristics of PEPCK which had been discovered two years previously in chicken liver. In 1957, PEPCK–ATP was reported to be present in a range of plant tissues (Mazelis and Vennesland, 1957).

In the 1960s it was established that PEPCK was likely to function in catalysing the gluconeogenic flux from stored lipid and protein in germinating seeds (Benedict and Beevers, 1961). Later work supported this (Leegood and ap Rees, 1978a). Although the enzyme from vertebrates was subject to much study in the 1960s (Utter and Kolenbrander, 1972), few studies were made of PEPCK from flowering plants during this decade. PEPCK was found to be present in the bundle sheath of the C4 plant *Panicum maximum*, in which it was thought likely to act as a decarboxylase and provide CO_2 for photosynthesis (Edwards *et al*., 1971). Subsequent studies suggested that C4 plants could be grouped into three types based on the decarboxylase employed in the bundle sheath (Gutierrez *et al*., 1974; Hatch *et al*., 1975). PEPCK is found in the leaves of some CAM plants, in which it functions to provide CO_2 for photosynthesis by decarboxylating organic acids during the day (Dittrich *et al*., 1973) and in grapes, in which it is likely to catalyse a gluconeogenic flux from organic acids during ripening (Ruffner and Kliewer, 1975). PEPCK was obtained in a highly purified (Hatch and Mau, 1977) and pure form (Burnell, 1986) from leaves of *P. maximum*, and its catalytic properties were characterised. The kinetic mechanism of PEPCK from flowering plants was studied, and it was concluded that the enzyme likely operates by a random sequential mechanism (Arnelle and O'Leary. 1992). In the late 1970s and early 1980s a number of groups established that, in flowering plants, PEPCK was located in the cytosol (Leegood and ap Rees, 1978a; Ku *et al*., 1980 and references therein). A number of detailed studies established the functioning of PEPCK in the C4 cycle (Hatch and Kagawa, 1976; Rathnam and Edwards, 1977; Hatch, 1979; Smith and Woolhouse, 1983, and references therein). It was later found that both PEPCK and mitochondrial NAD–malic enzyme acted as decarboxylases in the bundle sheath of this group of C4 plants,

and that the action of NAD–malic enzyme was important in the provision of ATP for the PEPCK reaction (Burnell and Hatch, 1988a, b). Subsequent studies further characterised the role of mitochondrial metabolism in the provision of ATP (Carnal *et al*., 1993; Agostino *et al*., 1996). Maize was found to use a different mechanism for decarboxylation of organic acids in the bundle sheath than was previously thought, and to use both chloroplastic NADP–malic enzyme and PEPCK (Walker *et al*., 1997; Furumoto *et al*., 1999; Wingler *et al*., 1999). This represented a fourth type of C4 plant, the existence of which had been suggested by the work of Gutierrez *et al*. (1974).

The first sequence of a cDNA encoding PEPCK from flowering plants was obtained from cucumber in 1994 (Kim and Smith, 1994). Since then, the sequence of PEPCK from many flowering plants has been obtained. In some species there is only one gene for PEPCK (Kim and Smith, 1994), while in others there is a small multigene family, members of which are expressed in different tissues (Finnegan *et al*., 1999). The evolutionary origins of this situation are unknown, as is and whether or not these gene products possess different properties. PEPCK from flowering plants was found to possess an extension of about 12 kDa at its N-terminus compared with PEPCK–ATP from other organisms of otherwise very similar sequence (Kim and Smith, 1994). Evidence is presented in this article that this N-terminal extension is likely to be homologous to the N-terminal region of PEPCK–GTP. The N-terminal extension was shown to be rapidly lost from the enzyme from flowering plants upon extraction (Kim and Smith, 1994; Finnegan and Burnell, 1995; Walker *et al*., 1995). This meant that all previous purifications of the enzyme from flowering plants were of a truncated form. PEPCK from a range of flowering plants was found to be phosphorylated at a site within the N-terminal extension both *in vitro* and *in vivo* (Walker and Leegood, 1995; Walker and Leegood, 1996). Phosphorylation of PEPCK both *in vitro* and *in vivo* lowers its affinity for both PEP and OAA, which is dependent on the concentration of OAA, PEP and adenylates and on the ratio of ATP:ADP (Walker *et al*., 2002). How this mechanism allows regulation of the activity in a wide range of plant tissues is described in this article. Chen *et al*. (2002) provided the first evidence that PEPCK from any organism possesses a high affinity for CO_2 when assayed under more physiological conditions. The implications of this are also discussed in this article. Chen *et al*. (2000) showed that it was highly unlikely that a gluconeogenic flux which utilised PEPCK occurred in senescing or starved leaves – a view which had been widely expressed. Although PEPCK had been found in a range of tissues from flowering plants in the 1950s (Mazelis and Vennesland, 1957), it was generally thought that the enzyme only functioned in gluconeogenesis and in the C4 and CAM photosynthetic CO_2-concentrating mechanisms. Over the last few years the presence of PEPCK in a wide range of tissues in flowering plants has been confirmed and extended (Kim and Smith, 1994; Walker *et al*., 1999; Chen *et al*., 2000; Walker *et al*., 2001). These studies have shown that PEPCK is widely distributed in flowering plants and is abundant in many tissues such as the

vasculature, trichomes, developing seeds and roots. In many of these tissues PEPCK may be a component of a mechanism that is involved in the regulation of intracellular pH (Walker *et al*., 2001). Further evidence for this is presented in this article and its proposed role in this mechanism is described.

III. DISTRIBUTION OF PEPCK

Two types of PEPCK are found, those which use only, or show higher activity with adenine nucleotides (PEPCK–ATP: EC 4.1.1.49), and those which use guanine, or sometimes inosine nucleotides (PEPCK–GTP: EC 4.1.1.32) as a substrate (Utter and Kolenbrander, 1972). In addition, the enzyme PEP carboxytransphosphorylase (EC 4.1.1.38), that appears to possess a similar catalytic mechanism but uses pyrophosphate instead of nucleoside triphosphates as a substrate, has been found in a small number of bacteria and protoctists (see Utter and Kolenbrander, 1972; Dawes, 1986). It is unknown whether this enzyme shows sequence similarity with PEPCK. The ATP-dependent enzyme has been found in flowering plants, ascomycetes, basidomycetes, brown algae, red algae, green algae, diatoms, dinoflagellates, kinetoplastids and many bacteria. The GTP-dependent enzyme has been found in mammals, birds, fish, insects, molluscs, flatworms, nematodes, *Euglena,* the chytrid *Neocallimastix frontalis* and many bacteria. The evolutionary origins of this distribution are unknown. For a description of the organisms mentioned in this article and modern view on their evolutionary relationships the reader is referred to Margulis and Schwartz (1998).

IV. STRUCTURE OF PEPCK

A. SEQUENCE COMPARISONS: RELATIONSHIP BETWEEN PEPCK–ATP AND PEPCK–GTP

Within the ATP- and GTP-groups there is high sequence similarity. In contrast, it is generally thought that there is no significant sequence similarity between the ATP- and GTP-dependent PEPCKs, and that the enzymes arose by convergent evolution. On the other hand, the ATP- and GTP-dependent enzymes possess similar catalytic properties, catalytic mechanisms and tertiary structures (Matte *et al*., 1997; Dunten *et al*., 2002). A careful alignment of the sequences of ATP- and GTP-dependent PEPCKs shows that over the entire sequence there are many residues and motifs at the same place in the sequence conserved (Fig. 1). These observations raise the question as to whether PEPCK–ATP and PEPCK–GTP arose by convergent or divergent evolution, and the question of the origin of the N-terminal extension possessed by the enzyme from flowering plants (Kim and Smith, 1994). The bacterial c-type cytochrome family is thought to have arisen by divergent evolution. The family shows large differences in size and overall sequence similarity, but there are conserved residues at

```
             1                                                    50
     tomato  .masng.vgn gefsfdnkrr tglpkiqtqk tedehnvvchd dsatpvkaqt
    flaveria .masng...n gnvagkgngr nglpkiqtqk ..rqngichd dssapvkvqt
 arabidopsis msagngnatn gdggfsfpkg pvmpkittga akrgsgvchd dsgptvnatt
    humancyt .......... .......... ..mp.pqlq. ....nglnl. .s.akv.vqg
      ratcyt .......... .......... ..mp.pqlh. ....ngldf. .s.akvi.qg
  chickencyt .......... .......... ..ma.pel.k tev.nii... .s..kvi.qq

             51                                                  100
     tomato  leelhsl.qk kksapttp.. ik.sphvfgv avseee..rq kqqlqsisas
    flaveria idelhsl.qr kksapttp.. ld.gvqgafa nfsede..rq kqqlqsisas
 arabidopsis idelhsl.qk krsapttp.. inqnaaaafa avseee..rq kiqlqsisas
    humancyt ..sldslpqa vreflennae lcqpdhihic dgseeengrl lgqmeeegil
      ratcyt ..sldslpqe vrkfvegnaq lcqpeyihic dgseeeygrl lahmqeegvi
  chickencyt .d.leslppq vrefiesnak lcqpesihic dgseeenkki ldimveqgmi

             101                                                 150
     tomato  .......... laslt..... .r.etgpkvv kgdparqae. ...tpkvqqp
    flaveria .......... laslt..... .r.etgpkvv rgdparrpe. ...tprvahg
 arabidopsis .......... laslt..... .r.esgpkvv rgdpaekktd gsttpayahg
    humancyt rrlkkydncw la.ltdprdv aries..ktv ivtq.eqr.d ...t..v..p
      ratcyt rklkkydncw la.ltdprdv aries..ktv iitq.eqr.d ...t..v..p
  chickencyt kklskyencw la.ltnprdv aries..ktv iitq.eqr.d ...t..i..p

             151                                                 200
     tomato  vhhhh.tp.a lnisdsglkf thilynlspa elyeqaikye kgsfit....
    flaveria pdhyf.tp.t faasdsslkf thvlynlspa elyeqaikye kgsfit....
 arabidopsis qhhsifspat gavsdsslkf thvlynlspa elyeqaikye kgsfit....
    humancyt ......ipkt gl...sqlgr wm.....s.e edf......e ka.fnarfpg
      ratcyt ......ipks gq...sqlgr wm.....s.e edf......e ka.fnarfpg
  chickencyt ......ipkt g..s.sqlgr wm.....s.e edf......e ka.fntrfpg

             201                                                 250
     tomato  .......... ..ssgal.at lsgaktg... .hsprdkrvv ...rdetted
    flaveria .......... ..ssgal.at lsgaktg... .rspkdkrvv ...rddvten
 arabidopsis .......... ..sngal.at lsgaktg... .raprdkrvv ...rdatted
    humancyt cmkgrtmyvi pfsmgplgsp ls..kigiel tdspy...vv asmrimtrmg
      ratcyt cmkgrtmyvi pfsmgplgsp l..akigiel tdspy...vv asmrimtrmg
  chickencyt cmqgrtmyvi pfsmgpigsp l..akigiel tdspy...vv asmrmmtrmg

             251                                                 300
     tomato  d..l.wwgkg spniemdeqt flinrerav. dylcslekvf vndqflnwdp
    flaveria e..l.wwgkg spniemdeqt flvnrerav. dylnslekvf vndqflnwdp
 arabidopsis e..l.wwgkg spniemdeht fmvnrerav. dylnslekvf vndqylnwdp
    humancyt tpvleavgdg .......e.. f.vkclhsvg cplp.lqkpl vnnwpcn..p
      ratcyt tsvlealgdg .......e.. f.ikclhsvg cplp.lkkpl vnnwacn..p
  chickencyt taalkalgng .......e.. f.vkclhsvg cplp.lkepl innwpcn..p

             301                                                 350
     tomato  nnr.ikvriv sarayhslfm hnmcirptpe eledfgtpdf tiynagq..f
    flaveria enr.ikvriv sarayhslfm hnmcirpspe elenfgtpdf tiynagq..f
 arabidopsis enr.ikvriv sarayhslfm hnmcirptqe elesfgtpdf tiynagq..f
    humancyt eltli..... ...a.h.l.. ......pdrr eiisfgsgyg gnsllgkkcf
      ratcyt eltli..... ...a.h.l.. ......pdrr eiisfgsgyg gnsllgkkcf
  chickencyt eltli..... ...a.h.l.. ......pdrr eiisfgsgyg gnsllgkkcf
```

Fig. 1. Comparison of the amino acid sequences of PEPCK–ATP and PEPCK–GTP. PEPCK–ATPs are from the flowering plants, tomato (AAG01894), *Arabidopsis* (NP-195500) and *Flaveria* (BAB43908). Cytosolic PEPCK–GTPs are human (P3558), chicken (P05153) and rat (P07379). Accession numbers are given in brackets.

```
                351                                                 400
     tomato     pcnrythymt sstsidinla rremvilg.t qyagemkkgl fgvmhylmpk
   flaveria     pcnrythymt sstsidlnlg rremvilg.t qyagemkkgl fgvmhylmpk
arabidopsis     pcnrythymt sstsvdlnla rremvilg.t qyagemkkgl fsvmhylmpk
   humancyt     al.rmasrla keeg...wla eh.mlilgit npege.kkyl ..........
     ratcyt     al.riasrla keeg...wla eh.mlilgit npegk.kkyl ..........
 chickencyt     al.riasria keeg...wla eh.mlilgit npege.kky. f.........

                401                                                 450
     tomato     rqilslhsgc nmgkegdval ffglsgtgkt tlstdhnryl .......igd
   flaveria     rqilslhsgc nmgkdgdval ffglsgtgkt tlstdhnryl .......igd
arabidopsis     rrilslhsgc nmgkdgdval ffglsgtgkt tlstdhnryl .......igd
   humancyt     .......... .......aaa fp..sacgkt nl.ammnpsl pgwkvecvgd
     ratcyt     .......... .......aaa fp..sacgkt nl.ammnptl pgwkvecvgd
 chickencyt     .......... .......aaa fp..sacgkt nl.ammnpsr pgwkiecvgd

                451                                                 500
     tomato     dehcwsdhgv snieggcy.a kcidlareke pdiwnai..k fgtvlenv.v
   flaveria     dehcwsdkgv snieggcy.a kcidlskeke pdiwnai..k fgtvlenv.v
arabidopsis     dehcwtetgv snieggcy.a kcvdlsreke pdiwnai..k fgtvlenv.v
   humancyt     di.awm.kfd ...aqghlra .......... ..i.npengf fg.vapgtsv
     ratcyt     di.awm.kfd ...aqgnlra .......... ..i.npengf fg.vapgtsv
 chickencyt     di.awm.kfd ...elgnlra .......... ..i.npengf fg.vapgtsi

                501                                                 550
     tomato     feehtrevdy tdkf...... .vtentraa. .ypieyi..p nakipcvgph
   flaveria     fdehtrevdy ldks...... .vtentraa. .ypieyi..p nakipcvgph
arabidopsis     fdehtrevdy sdks...... .vtentraa. .ypiefi..p nakipcvgph
   humancyt     ...ktnpnai ktiqkntift nvae.tsdgg vyw.egidep la..sgvtit
     ratcyt     ...ktnpnai ktiqkntift nvae.tsdgg vyw.egidep la..pgvtit
 chickencyt     ...ktnpnai ktifkntift nvae.tsdgg vyw.egidep lp..pgvtlt

                551                                                 600
     tomato     .pknvillac dafgvlppvs klnl.a.qtm yhfi...sgy talvagtedg
   flaveria     .pknvillac dafgvlppvs klsl.a.qtm yhfi...sgy talvagteeg
arabidopsis     .ptnvillac dafgvlppvs klnl.a.qtm yhfi...sgy talvagtedg
   humancyt     swknkewsse dgepcahpns rfctpasqcp iidaawespe gvpiegiifg
     ratcyt     swknkewrpq deepcahpns rfctpasqcp iidpawespe gvpiegiifg
 chickencyt     swknkdwtpd ngepcahpns rfctpasqcp imdpawespe gvpiegiifg

                601                                                 650
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   flaveria     vkepratfsa cfgaafimlh ptky.aeml. .atkmekh.g atgwlvntgw
arabidopsis     ikeptatfsa cfgaafimlh ptky.aaml. .aekmksq.g atgwlvntgw
   humancyt     grrpagvplv ..yealswqh gvfvgaamrs eataaaehkg kiimhdpfam
     ratcyt     grrpagvplv ..yealswqh gvfvgaamrs eataaaehkg kvimhdpfam
 chickencyt     grrpagvplv ..yeafnwqh gvfigaamrs eataaaehkg kiimhdpfam

                651                                                 700
     tomato     s.ggsygsgs riklaytrkn idaihsgdll kaeykktev. .......fgl
   flaveria     s.ggsygags rmklaytrki idaihsgkll nanykktev. .......fgl
arabidopsis     s.ggsygvgn riklaytrki idaihsgsll kanykktei. .......fgf
   humancyt     rpffgynfgk ylahwlsmaq hpaaklpkif hvnwfrkdke gkflwpgfg.
     ratcyt     rpffgynfgk ylahwlsmah rpaaklpkif hvnwfrkdkn gkflwpgfg.
 chickencyt     rpffgynfgk ylahwlsmah rpaaklprif hvnwfrkdsq gkflwpgyg.
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Fig. 1. *Continued*

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             701                                              750
     tomato  eiptalegvp seildpvntw pdkkah..kd t.ll.klgg. .....lf..r
   flaveria  eiptevegvp seildpvntw snkkay..ke t.ll.klag. .....lf..k
arabidopsis  eipteiegip seildpvnsw sdkkah..kd t.lv.klgg. .....lf..k
   humancyt  ensrvlewmf nridgkastk ltpigyipke dalnlkglgh inmmelfsis
     ratcyt  ensrvlewmf griegedsak ltpigyvpke dalnlkglgd vnveelfgis
 chickencyt  ensrvlewmf nriqgkasak staigyipad talnlkgled inltelfnis

             751                                  791
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   flaveria  nnf...evfv nhkigkddk. ..ltqei... .laagp.nf
arabidopsis  knf...evfa nhkigvdgk. ..lteei... .laagp.if
   humancyt  kefwekeved iekyledqvn adlpceiere ilalkqrisq m
     ratcyt  kefwekevee idkyledqvn adlpyeiere lralkqrisq m
 chickencyt  kefwekevee ikqyfegqvn adlpyeiere llalemrikq l
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Fig. 1. *Continued*

positions in their primary sequence that are critical for their higher-order structures. Crystallography showed that these proteins all possessed a similar structure that was proposed to have been conserved during the evolution of this protein family (Salemme, 1977). A number of proteases are thought to have arisen by convergent evolution; these possess a similar catalytic mechanism and geometry of residues at their active sites. However, these residues occupy different positions in the primary sequence (Creighton, 1993). These observations argue very strongly against convergent evolution of PEPCKs and suggest a common ancestor. The large difference in overall sequence similarity between the ATP- and GTP-dependent PEPCKs and their distribution in living organisms suggests that divergence occurred relatively early in the evolutionary process. In terms of understanding the evolutionary history of PEPCK, it will be interesting to see how a sequence comparison of PEPCK from bacteria and protoctists compares with the proposed evolutionary relationships of these groups.

PEPCK–ATPs possess high sequence similarity over the central 80% of the protein; however, the enzyme from flowering plants possesses an extension of about 12 kDa at its N-terminus compared with PEPCK–ATP from other organisms (Kim and Smith, 1994; Finnegan and Burnell, 1995). A comparison of the sequence of PEPCKs from flowering plants with those of GTP-dependent PEPCKs show that both contain a ‘N-terminal extension’; this contains a number of groups of conserved residues at the same position in the sequence (Fig. 1). This strongly suggests that these ‘N-terminal extensions’ are homologous and that it was lost from non-plant ATP-dependent PEPCKs. The N-terminal extension of the plant enzyme is not required for catalysis but for regulation (Walker *et al.* 1995; Walker and Leegood, 1996; Walker *et al.*, 2002). In prokaryotes and yeast, regulation of PEPCK–ATP, by changes in its abundance in response to factors such as sugar availability, is important (Utter and Kolenbrander, 1972). A possibility is that regulation of the enzyme by mechanisms that required the N-terminal extension was not so important in these unicellular organisms, and was lost. This is supported by the observation that in

flowering plants, which possess the C4-photosynthetic CO_2-concentrating mechanism, the enzyme expressed in leaves possesses a truncated N-terminal extension which is often not phosphorylated (Walker and Leegood, 1996). This is because in this tissue, phosphorylation is not required to regulate the enzyme. The likely reason for this is described later in this article, in section VIII.B.1.

B. TERTIARY STRUCTURE

The crystal structure of PEPCK has been determined for PEPCK–ATP from the bacterium *Escherichia coli* (Matte *et al*., 1996) and the protoctist *Trypanosoma cruzi* (Trapani *et al*., 2001), and for PEPCK–GTP from humans (Dunten *et al*., 2002). The fold of the enzymes is very similar, the major difference being that at the active site the pocket that binds the nucleotide substrates base is different for PEPCK–GTP (Dunten *et al*., 2002). The enzyme from at least some flowering plants, unlike the enzyme from *E. coli* and *T. cruzi*, utilises GTP with about 30% of the efficiency of ATP (Hatch and Mau, 1977; Burnell, 1986; R. P. Walker, unpublished observations). It will be interesting to know the structural basis for this. PEPCK is composed of two similar-sized domains, termed the N- and C-terminal domains. These are separated by a deep cleft in which the active site is located. At least for PEPCK–ATP the protein undergoes a conformational transition upon binding the nucleoside triphosphate. This is not the result of a large change in conformation, but due to changes in the torsion angles of the polypeptides in the three regions that connect the two domains. This results in a hinge-like movement through 20°, which positions active-site residues and partially buries the active-site cleft. This may serve to create a favourable environment for catalysis (Matte *et al*., 1997). At the active site the A(G)TP–Mg^{2+} binding site contains a phosphoryl binding loop or P-loop, which interacts with the phosphoryl oxygen atoms of ATP. The P-loop is present in many ATP- or GTP-binding proteins. However, the fold of the P-loop is distinct from that described for any protein so far (Matte *et al*., 1997). PEPCK does not possess a overall degree of structural similarity with any known protein, and may represent the first member of a new structural family (Matte *et al*., 1997). For a detailed description of this topic and the active site, the reader is referred to Matte *et al*., 1997; Trapani *et al*., 2001; Dunten *et al*., 2002).

C. QUATERNARY STRUCTURE

PEPCK–ATP from bacteria and PEPCK–GTP are monomers. In contrast, PEPCK–ATP from kinetoplastids is a dimer (Trapani *et al*., 2001 and references therein). In yeast, PEPCK is a tetramer (Tortora *et al*., 1985). In flowering plants, PEPCK is reported to be either a tetramer (Walker *et al*., 1995) or a hexamer (Burnell, 1986) on the basis of gel-filtration chromatography. This discrepancy needs to be addressed, and it should be noted that anomalous behav-

iour of PEPCK–GTP using this technique has been reported for some column packings (Hebda and Nowak, 1982a). There is no evidence for dissociation of the subunits during purification, which occurs in the case of PEPC (see Chollet *et al*., 1996). The interaction and orientation of the subunits have been studied by crystallography for the enzyme from *T. cruzi* (Trapani *et al*., 2001). Monomer–monomer interaction is between only the N-terminal domains, and the area of interaction is small; this leaves the two C-terminal domains well separated. This arrangement would allow an independent access of substrates and would not hinder the movement of the two domains of each subunit on binding ATP (Trapani *et al*., 2001). It would be interesting to know how the subunits of PEPCK from flowering plants are arranged and what change phosphorylation brings about.

D. CATALYTIC MECHANISM

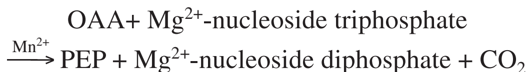
The catalytic mechanism and active-site structure of PEPCK has been reviewed recently, and the reader is referred to these reviews for a detailed account (Hanson and Patel, 1994; Matte *et al*., 1997; see also Trapani *et al*., 2001; Dunten *et al*., 2002). The similarities in overall amino acid sequence, tertiary structures, active site organisation and the possession of similar motifs involved in substrate interaction suggests that PEPCK–ATP and PEPCK–GTP possess a similar catalytic mechanism. The order of binding of substrates to the enzyme is a matter of controversy, and different mechanisms have been proposed for PEPCK from different sources. The enzyme from chicken liver mitochondria and flowering plants binds substrates in any order (Hebda and Nowak, 1982b; Arnelle and O'Leary 1992). It is uncertain whether the reaction occurs in one step (concerted) or two steps (sequential); opinion seems to favour the latter (Arnelle and O'Leary, 1992; Matte *et al*., 1997). Carbon dioxide and not bicarbonate is the substrate for the enzyme (see Utter and Kolenbrander, 1972; Arnelle and O'Leary, 1992), and a number of lines of evidence have suggested or shown that the enzyme possesses a binding site for CO_2 (Hebda and Nowak, 1982b; Cheng and Nowak, 1989; Arnelle and O'Leary, 1992). PEPCK requires two metal ions for activity; one ion is required because metal ion–nucleotide is the substrate – this ion is likely to be Mg^{2+} *in vivo* (Ash *et al*., 1990). PEPCK possesses a binding site, within the active site, for the second ion, which is thought to be Mn^{2+} *in vivo* (see Hebda and Nowak, 1982b). The role of this ion in catalysis is uncertain (Matte *et al*., 1997).

V. THE REACTIONS CATALYSED AND THEIR PROPERTIES

In general the catalytic properties of PEPCKs from diverse sources are surprisingly similar. The major established difference is their nucleotide requirement.

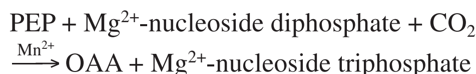
A. DECARBOXYLATION OF OAA TO PEP

This reaction is a component of a number of metabolic processes.



$$\text{OAA} + \text{Mg}^{2+}\text{-nucleoside triphosphate} \xrightarrow{\text{Mn}^{2+}} \text{PEP} + \text{Mg}^{2+}\text{-nucleoside diphosphate} + \text{CO}_2$$

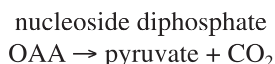
B. CARBOXYLATION OF PEP TO OAA



$$\text{PEP} + \text{Mg}^{2+}\text{-nucleoside diphosphate} + \text{CO}_2 \xrightarrow{\text{Mn}^{2+}} \text{OAA} + \text{Mg}^{2+}\text{-nucleoside triphosphate}$$

In flowering plants and vertebrates PEPCK is generally thought to act only as a decarboxylase. This was thought to be because its K_m for CO_2 was about 100 times greater than the concentration of CO_2 within a cell. However, recent work showed that, at least for the enzyme from flowering plants, this low affinity is an artefact of the conditions used to assay the enzyme *in vitro* (Chen *et al*., 2002). The reason why PEPCK does not generally act as a carboxylase in most tissues of flowering plants is not that the enzyme possesses a low affinity for CO_2, but that ATP is a potent inhibitor of the carboxylase activity of PEPCK (fig. 3 in Walker *et al*., 2002). At the concentration of PEP, OAA, ATP and ADP found in most plant cells, the carboxylase activity of PEPCK would be much less than that of the decarboxylase activity. However, in some organisms, such as parasitic worms in the gut (PEPCK–GTP), and in brown algae (PEPCK–ATP), the enzyme functions as a carboxylase. Similarly, in kinetoplastids PEPCK may function as a carboxylase *in vivo* under certain conditions (Urbina, 1987). It has been reported that in the case of PEPCK–GTP from the gut worm *Ascaris suum*, one contributory factor is that the enzyme possesses a low affinity for OAA (Saz, 1981 and references therein). However, it is also thought that the concentration of metabolites in the cell plays a major role (Rohrer *et al*., 1986). In the case of PEPCK–ATP from brown algae and kinetoplastids this is not because the enzyme is less susceptible to inhibition by ATP (Weidner and Küppers, 1982; Urbina, 1987). It is likely that in these organisms the *in vivo* concentration of OAA, PEP and adenylates enhances the carboxylase activity relative to the decarboxylase activity.

C. DECARBOXYLATION OF OAA TO PYRUVATE



$$\text{OAA} \xrightarrow{\text{nucleoside diphosphate}} \text{pyruvate} + \text{CO}_2$$

This reaction is catalysed by PEPCK–GTP from a number of sources, and PEPCK–ATP from yeast (Utter and Kolenbrander, 1972 and references therein).

This reaction is not catalysed by the enzyme from the few flowering plant species in which it has been investigated (Hatch and Mau, 1977; Burnell, 1986). Interestingly, mutation of Asp268 to Asn of PEPCK–ATP from *E. coli* maintains OAA decarboxylase activity but abolishes phosphoryl transfer (Matte *et al.*, 1997 and references therein). The physiological role of this reaction, if any, is unknown.

D. SPECIFIC ACTIVITY

In general the specific activity (units of activity per mg protein) of PEPCK–ATP is about 30–60 for both the carboxylation and decarboxylation reactions when measured under conditions that measure maximum activity at 25°C (Burnell *et al.*, 1986; Arnelle and O'Leary, 1992; Hunt and Köhler, 1995; Walker *et al.*, 2002). Lower specific activities are usually reported for PEPCK–GTP. However, when measured under conditions that result in maximum catalytic velocity, the specific activity of PEPCK–GTP from rat liver cytosol is similar to that of PEPCK–ATP (Colombo *et al.*, 1978). The value of specific activity is greatly altered by the oxidation state of –SH groups on the enzyme, and the metal ions present. For these reasons it is difficult to say whether there is any difference in the specific activity of PEPCK from different organisms.

E. NUCLEOSIDE PHOSPHATE SPECIFICITY

In general, two classes of PEPCK are recognised: those which are active with adenine nucleotides and those which are active with guanine or inosine nucleotides (Utter and Kolenbrander, 1972). However, the enzyme from at least some flowering plants, although showing higher activity with ATP (at least in the decarboxylation reaction) can use GTP which gives about one-third of the velocity. A number of other nucleotides also act as substrates (Hatch and Mau, 1977; Burnell, 1986). The point that some PEPCKs may show activity with both ATP and GTP was raised by Utter and Kolenbrander (1972).

F. METAL REQUIREMENTS

This is complex subject because a number of interactions are involved. PEPCK requires two cations for catalysis: one is required to bind to a site on the enzyme (although its role in catalysis is uncertain) the other is required because the metal–nucleotide is the substrate for the enzyme (Utter and Kolenbrander, 1972; Lee *et al.*, 1981; Ash *et al.*, 1990; Matte *et al.*, 1997). In general, PEPCK from different sources shows the highest activity with Mn^{2+} as the cation bound to the enzyme – a role it is thought to fulfil *in vivo*. In contrast, Mg^{2+}-nucleotide is thought to be the substrate *in vivo*. PEPCK often requires micromolar concentrations of Mn^{2+} and millimolar concentrations of Mg^{2+}. However, depending on the source of the enzyme, a number of other cations can sometimes fulfil these

roles in *in vitro* assay. However, the catalytic velocity and the substrate affinities are altered, and these effects differ for the carboxylase and decarboxylase directions (table 1 in Lee *et al.*, 1981 and references therein). Lee *et al.* (1981) reported that for the decarboxylase activity of PEPCK–GTP from chicken liver mitochondria, low concentrations of Co^{2+}, Fe^{2+}, Zn^{2+} and Cd^{2+} could substitute for Mn^{2+} in the presence of Mg^{2+}, and that 1.6 mM Mg^{2+} alone supported the reaction. Catalytic velocity was between 35 and 50% of that with Mn^{2+}/Mg^{2+}. In the carboxylation reaction, Mg^{2+} alone gave only 2% of the reaction rate with Mn^{2+} also present, and only Co^{2+} could substitute for Mn^{2+}. PEPCK–ATP from flowering plants appears to have an absolute requirement for Mn^{2+} (Burnell, 1986; Chen *et al.*, 2002), although this needs more detailed investigation. In contrast, for PEPCK–ATP from *Trypanosoma brucei*, Co^{2+} could substitute to some extent for Mn^{2+}, but only in the carboxylation direction (Hunt and Köhler, 1995). For PEPCK–ATP from flowering plants, activity in both carboxylation and decarboxylation directions is inhibited by about 50% when assayed in the presence of both millimolar concentrations of Mn^{2+} and Mg^{2+} compared with Mn^{2+} alone (Burnell, 1986; Chen *et al.*, 2002). This inhibition does not happen for the enzyme from *T. brucei* (Hunt and Köhler, 1995). The enzyme from flowering plants appeared to require a high concentration of Mn^{2+} for activity only in the carboxylation direction; however, the addition of 150 mM 2-mercaptoethanol to the assay restored activity in the presence of low concentrations of Mn^{2+} and millimolar concentrations of Mg^{2+}. It appears that oxidation of a –SH group occurs only in the carboxylation assay, and only if low concentrations of Mn^{2+} are present (Chen *et al.*, 2002). Therefore, in some cases the oxidation of –SH groups might play a role in determining the cation requirement. Similarly, Bentle and Lardy (1977) reported changes in the response to cations upon purification. It seems that the response of PEPCK to cations is not so much determined by whether it is an ATP- or GTP-type. An important point is that the affinity of PEPCK for its substrates is greatly altered by the concentration and type of cations present in *in vitro* assay (Lee *et al.*, 1981; Chen *et al.*, 2002). To understand the properties of PEPCK within the cell it is important to use physiological concentrations of these cations.

G. SUBSTRATE AFFINITIES

The marked differences between the published values of K_m for OAA were highlighted by Utter and Kolenbrander (1972), and they reported that these values may be dependent on the concentration of Mn^{2+} and Mg^{2+} in *in vitro* assay. The K_m for OAA and other substrates are dependent on these, and a number of other factors, in *in vitro* assay (see, for example, Lee *et al.*, 1981; Hebda and Nowak, 1982b; Chen *et al.*, 2002; Walker *et al.*, 2002). This means that a comparison of reported K_m values in many cases is very difficult because assays were done under different conditions. Another factor is that the properties of PEPCK are sometimes altered upon extraction and purification.

H. AFFINITY FOR CO_2

PEPCK, at least from flowering plants, does not appear to have a low affinity for CO_2. Most studies in which the affinity of CO_2 has been determined used conditions in *in vitro* assays very different from those within the cell. When Mn^{2+} and Mg^{2+} concentrations and the ATP:ADP ratio approximate those within the cell, the affinity of PEPCK for CO_2 is similar to that of ribulose bisphosphate carboxylase (RuBisCO) (Chen *et al*., 2002). It is interesting to note that the affinity of RuBisCO for CO_2 was originally thought to be low; this was also a result of unphysiological *in vitro* assay conditions (Jensen and Bahr, 1977). It should be noted that neither phosphorylation of the N-terminal extension nor its loss alter the affinity of PEPCK from flowering plants for CO_2. It will be interesting to see whether this result is confirmed by studies of PEPCK from other sources.

I. EFFECTORS

In general, the presence of most metabolites has little effect on PEPCK activity (Utter and Kolenbrander, 1972). However, there are exceptions. The catalytic velocity is greatly altered by the presence of different nucleotides. For PEPCK–ATP the carboxylase activity has been reported to be inhibited by ATP in a similar way in a wide range of organisms (Weidner and Küppers, 1982; Urbina, 1987; Hunt and Köhler, 1995). At physiological ratios of ATP to ADP, the carboxylase activity of PEPCK is greatly reduced (Walker *et al*., 2002). Similarly, the carboxylase activity of PEPCK–GTP is inhibited by ATP (Rohrer *et al*., 1986). The decarboxylase activity of PEPCK–ATP is inhibited by ADP (Burnell, 1986) but the amount of inhibition is much less than that of the carboxylase activity by ATP (Walker *et al*., 2002). Therefore, this does not appear to be a result of mass action. Importantly, the ratio of ATP to ADP in *in vitro* assay greatly alters not only the catalytic velocity of PEPCK but its affinity for its substrates. To understand how PEPCK might behave in the cell it is important to use physiological concentrations, and ratios of ATP to ADP in *in vitro* assays. A number of sugar phosphates inhibit PEPCK–ATP from flowering plants by up to about 70% at a concentration of 5 mM, if 0.5 mM Mn^{2+} is the only cation present (Hatch and Mau, 1977; Burnell, 1986). Inhibition is relieved by increasing the concentration of Mn^{2+}, and is not seen in the presence of more physiological concentrations of Mn^{2+} and Mg^{2+} (Burnell, 1986). This effect is probably not important *in vivo* (see Carnal *et al*., 1993). Titheradge *et al*. (1992) reported that physiological concentrations of α-ketoglutarate inhibit cytosolic PEPCK–GTP from liver.

J. pH OPTIMA

In general, the pH optimum of the carboxylation reaction is thought to be lower than that of the decarboxylation direction (Utter and Kolenbrander, 1972).

However, this may be dependent on the conditions used in *in vitro* assay. It is possible that if *in vitro* assay conditions approximate more closely those in the cell, then the results obtained would be different. Small changes in pH *in vivo* could potentially have a large effect on PEPCK activity, as has been proposed for PEPC. For example, in *Sorghum vulgare* leaves, a fall in cytosolic pH from 7.2 to 7.1 has been calculated to reduce the activity of PEPC six-fold at saturating light (Echevarria *et al*., 1994). This is a result of a number of interactions.

K. SOME DIFFICULTIES ENCOUNTERED IN STUDYING THE CATALYTIC PROPERTIES OF PEPCK

Because of the many interactions that occur between substrates, products and metal ions, studies that address how the enzyme might be regulated in the cell should be made under conditions that approximate those found *in vivo*. This is particularly difficult because of the low concentration of OAA (5–20 μM) in the cytosol of most cells (see Titheradge *et al*., 1992; Agostino *et al*., 1996; Petersen *et al*., 2001).

The enzyme from flowering plants is subject to modification by proteolysis upon extraction, which results in loss of a 12 kDa fragment (Kim and Smith, 1994; Finnegan and Burnell, 1995; Walker *et al*., 1995). The severity of this problem is dependent on the plant species and the tissue used, and in general takes several hours at pH 7.0 for a complete loss to occur. In general, increasing the pH of the extraction buffer affords the best protection (Walker *et al*., 1995). However, loss of the N-terminal extension does not alter the catalytic velocity of PEPCK, under conditions which measure its maximum activity but does change its regulatory properties (Walker *et al*., 2002). This means that all purifications of PEPCK from flowering plants before 1995 have been of this truncated form. It was not possible to know this because the cDNA had not then been sequenced, and an antibody was not available; therefore, the molecular weight of the enzyme in rapidly prepared extracts could not be compared with that of the pure enzyme by immunoblots of SDS–PAGE (sodium dodecyl sulphate, polyacrylamide gel electrophoresis) gels.

Loss, or modification, of activity can occur during extraction, purification, or assay that results from an oxidation of –SH groups (Utter and Kolenbrander, 1972; Colombo *et al*., 1978). The –SH group involved differs between PEPCKs (Cardemil *et al*., 1990; Bazaes *et al*., 1993; Trapani *et al*., 2001, and references therein). There is general agreement that these residues are not involved in catalysis (Trapani *et al*., 2001, and references therein). In cytosolic PEPCK–GTP Cys288 is involved and the presence of GTP protects the enzyme from modification (Lewis *et al*., 1989; Holyoak and Nowak, 2001). Crystallography studies show that when nucleotide is bound to the enzyme, the –SH group of this residue is less exposed to the solvent, which thus affords protection. Modification of this residue, which is a component of the P-loop, is likely to prevent the P-loop acquiring a conformation required for productive GTP-binding (Dunten *et al*., 2002). In yeast, the residue involved is Cys364, and the

presence of ADP plus Mn^{2+} completely protects the enzyme from inactivation by thiol-directed agents (Alvear *et al*., 1992). This residue is located in the adenine-binding site (Linss *et al*., 1993). The enzyme from flowering plants, unlike that from *Trypanosoma brucei* and *T. cruzi*, also contains this residue. This may explain why inactivation of PEPCK in crude extracts of some flowering plant tissues is protected by the presence of these compounds (Walker *et al*., 1997). Inactivation of PEPCK may occur during enzyme assay. For example, the enzyme from the flowering plant *Panicum maximum* requires either millimolar concentrations of Mn^{2+} or a very high concentration of 2-mercaptoethanol in the decarboxylase assay to be active; in the decarboxylation assay these conditions are not required (Chen *et al*., 2002).

From the foregoing, it is clear that many factors can influence the properties of PEPCK, and studies that address whether PEPCK from different plants, or different forms from the same plant, possess different properties will have to be done carefully, because many factors may result in apparently different properties and misleading conclusions. For these reasons Lepiniec *et al*. (1993) proposed that there was no conclusive evidence that PEPC from either different tissues or plants possessed different properties. However, recent detailed studies have clearly shown the opposite (Westhoff *et al*., 1997).

VI. SUBCELLULAR LOCATION

In vertebrates there are two forms of PEPCK: one is located in the cytosol, the other in the mitochondrion (Nordlie and Lardy, 1963). Although these forms arise from different genes, they possess similar catalytic properties (see Weldon *et al*., 1990). The ratio of mitochondrial to cytosolic PEPCK in liver is dependent on the species, and this correlates with the importance of lactate as a gluconeogenic substrate in that species. For example, birds produce large amounts of lactate in their muscles during flight, and these contain a much greater proportion of mitochondrial PEPCK. This is thought to be because metabolism of imported lactate to pyruvate by lactate dehydrogenase produces NADH in the cytosol. In contrast, when amino acids are used in gluconeogenesis, NADH is not usually produced in the cytosol as a direct result of their metabolism. This situation requires cytosolic malate dehydrogenase and cytosolic PEPCK in order to produce NADH in the cytosol. It should be noted that mitochondria from vertebrates, unlike those from flowering plants, do not possess a carrier for OAA at the inner mitochondrial membrane. In vertebrates the malate-aspartate shuttle cannot provide the cytosol with NADH because mitochondrial aspartate translocation is unidirectional (see Watford *et al*., 1981). In liver, NADH so produced is used by glyceraldehyde-3-phosphate dehydrogenase in gluconeogenesis (for a review see Hanson and Patel, 1994).

In the kinetoplastids *T. cruzi* and *T. brucei*, PEPCK is located in the glycosome (Kueng *et al*., 1989; Parsons and Smith, 1989). In flowering plants, only one form of PEPCK is known, located in the cytosol. This was determined by

subcellular fractionation studies of a range of tissues (Leegood and ap Rees, 1978a; Ku *et al*., 1980 and references therein). Earlier studies reported PEPCK to be located in the particulate fraction, from which localisation in the chloroplast or mitochondrion was implied. These results were incorrect, and resulted from problems with the fractionation technique (Ku *et al*., 1980). Recently PEPCK has been proposed to be present in the chloroplast of diatoms on the basis of subcellular fractionation (Reinfelder *et al*., 2000); however, more work is required to substantiate this (Johnston *et al*., 2001).

VII. THE PEPCK GENES

In the early to mid-1980s the first sequences of cDNAs and genes for PEPCK were obtained – these were for PEPCK–GTP from vertebrates (Hanson and Patel, 1994 and references therein). The first sequence of PEPCK–ATP was obtained for yeast in 1988 (Stucka *et al*., 1988), and the first cDNA sequence of PEPCK–ATP from flowering plants in 1994 (Kim and Smith, 1994). The partial genomic organisation of a small multigene family for PEPCK in the flowering plant, *Urochloa panicoides*, was reported in 1999 (Finnegan *et al*., 1999). The *Arabidopsis* genome-sequencing project has shown that three possible PEPCK genes are present (see Finnegan *et al*., 1999). In vertebrates the mitochondrial and cytosolic forms of PEPCK are encoded by separate nuclear genes (see Hanson and Patel, 1994). The mitochondrial forms from different species have a higher sequence similarity to each other than to that of cytosolic PEPCK from the same species. The same comment applies to the cytosolic forms. The sequence similarity between mitochondrial and cytosolic PEPCK is about 55%. These two forms of PEPCK may have evolved by gene duplication, possibly in response to the evolution of the Cori cycle (the transport of lactate from muscles to liver, gluconeogenesis from lactate in liver and transport of glucose to muscle) as the anatomy of animals became more complex (Weldon *et al*., 1990). The rat and human genes for cytosolic PEPCK and the human gene for mitochondrial PEPCK have the same gene structure of ten exons and nine introns. This further supports the proposal of gene duplication rather than incorporation of PEPCK from a bacterial genome (Modaressi *et al*., 1998). The intron/exon structure of the genes for PEPCK from *Arabidopsis* is different from vertebrates and from each other (Z.-H. Chen, R. P. Walker, unpublished observations). In the vertebrates studied so far, there is, as far as we are aware, only one gene for mitochondrial and one gene for cytosolic PEPCK (Hanson and Patel, 1994; Modaressi *et al*., 1998). In *Trypanosoma brucei* there is thought to be only one gene, whereas in *T. cruzi* there are at least two (Linss *et al*., 1993). In flowering plants the number of genes present is dependent on the species. In cucumber and tomato there is thought to be only one (Kim and Smith, 1994; Bahrami *et al*., 2001). In rapeseed (*Brassica napus*) (Sáez-Vásquez *et al*., 1995) and the grass *U. panicoides* (Finnegan *et al*., 1999) there is a small multigene family with at least four members in each. The

origins and any functional significance of this are unknown. It should be noted that at least half the species of flowering plant are not diploid but polyploid, and many of these have arisen by hybridisation between different species and genera (Raven *et al*., 1992). In *U. panicoides* each gene is capable of being transcribed, and the mRNAs for two of the genes are more abundant in leaves, and those for the other two, are more abundant in the roots (Finnegan *et al.*, 1999). Sequencing of PEPCK protein from leaves showed that the mRNA arising from the two genes transcribed in leaves was translated (Finnegan *et al.*, 1999).

VIII. REGULATION

The reaction catalysed by PEPCK lies at an important metabolic branchpoint and partly for this reason, although there are others such as the provision of CO_2 for photosynthesis, flux through the reaction is potentially capable of influencing a number of metabolic processes. Flux through this reaction is subject to strict control and one way in which this is achieved is by changes in the abundance of PEPCK protein. In multicellular organisms, such as vertebrates and flowering plants, PEPCK is only present in certain types of cell, and in many of these only under certain conditions. In micro-organisms the presence of PEPCK is often dependent on environmental factors, the importance of which depends on the species. A second way in which flux through the PEPCK reaction is controlled is that the catalytic velocity of a given amount of PEPCK protein can be altered *in vivo*. An important factor in this is likely to be changes in OAA concentration, and in flowering plants phosphorylation of PEPCK reduces its catalytic velocity at a given concentration of OAA. A third control is by substrate cycles: an example, in flowering plants and some bacteria, is one involving PEPC. These different control mechanisms will now be reviewed.

A. CHANGES IN ABUNDANCE OF PEPCK PROTEIN

1. Vertebrates

In vertebrates the abundance of cytosolic PEPCK is greatly altered by a number of stimuli such as nutrition and hormones. This was known in the 1960s (see Utter and Kolenbrander, 1972). In contrast, the abundance of mitochondrial PEPCK is fairly constant in most situations (Weldon *et al*., 1990), although there are exceptions (Hanson and Patel, 1994). In mammals the factors that bring about changes in the abundance of cytosolic PEPCK have been the subject of much study, and this system has been used as a model to study the metabolic control of gene transcription. This subject has been described in detail recently, and the reader is referred to Hanson and Patel (1994) and Hanson and Reshef (1997) for detailed accounts. In mammals, PEPCK is present in a number of tissues and in some its physiological role is unclear (Hanson and Reshef, 1997, and references therein). In mammals PEPCK is important in gluconeogenesis in

the liver and kidney, and in glyceroneogenesis in adipose tissue and the liver. In kidney, gluconeogenesis is also associated with the regulation of acid–base balance. The abundance of PEPCK in a tissue in which it is expressed is controlled by a large number of stimuli, and their relative importance depends on the type of cell and the developmental and physiological status of the animal. In general, in liver, hormonal signals related to fasting lead to the appearance of cytosolic PEPCK while an increase in glucose in the blood leads to its disappearance. In kidney an important signal leading to its appearance is acidosis. In liver the inhibitory action of insulin and the stimulatory action of cyclic AMP (cAMP) (mediated by glucagon) are major factors. How these signals are perceived and transduced is a complex subject, and the reader unfamiliar with this is referred to Lodish *et al*. (1995) and Voet and Voet (1995) for a basic overview. The following simplified example illustrates some features of how these signals are transduced. In the case of PEPCK the promoter contains a cAMP regulatory element (CRE) – the transcription factor that binds to this is known as CREB, phosphorylation of CREB determines its ability to bind to CRE. The phosphorylation of CREB is determined by a number of factors, one of which is the abundance of cAMP-dependent protein kinase in the nucleus. An important factor in determining this is the concentration of cAMP in the cytosol, which activates cAMP-dependent protein kinase and leads to its transport into the nucleus. The concentration of cAMP in the cytosol is increased by glucagon, for example, binding to its receptor in the plasma membrane.

The abundance of PEPCK in liver is set by the rate of transcription of the gene and the rate of turnover of its mRNA. Elevated mRNA leads directly to an increase in the synthesis of the protein. The promoter of cytosolic PEPCK contains at least a dozen regulatory elements, and these are responsible for the complex pattern of transcription. Analysis of the binding of proteins from the nuclei of different types of cell to the promoter of PEPCK shows that nuclear protein (transcription factors) from different tissue binds to some common and some different elements. This is dependent on the complement of transcription factors present in the tissue in question. The physiological importance of these response elements has been studied using transgenic mice in conjunction with constructs containing various alterations to the promoter. These studies showed that the relative importance of these response elements is dependent on the cell type in question. The picture that emerged is that different elements are used to drive expression of the gene in different tissues and situations. Elements that are stimulatory in one may be inhibitory in others. In each situation an element may acquire a different role suited to the complement of transcription factors present (Hanson and Reshef, 1997).

2. Micro-organisms

Like vertebrate cells, the abundance of PEPCK in micro-organisms is responsive to changes in the composition of medium in which they are bathed. Which factors are important depends on the organism in question. In yeast (*Saccharomyces*

cerevisiae) and many bacteria, the presence of PEPCK is repressed by sugars (see Utter and Kolenbrander, 1972). However, this does not happen in species such as those which employ PEPCK as a carboxylase in fermentation pathways (Podkovyrov and Zeikus, 1993). In the fungus *Amanita muscaria*, PEPCK is also thought to act as a carboxylase, and its abundance is not decreased by sugars (Wingler *et al*., 1996). This fungus forms a mycorrhizal association with the roots of the Norway spruce. Mycorrhizae occur in many species of vascular plants and are important in the acquisition of inorganic nutrients from the soil (for a description see Raven *et al*., 1992). *A. muscaria* imports sugars from the root and nitrogen compounds from the soil; it is proposed that anaplerotic replenishment of the Krebs cycle, which is necessary for the assimilation of nitrogenous compounds, is performed by PEPCK in this species (Wingler *et al*., 1996). In photosynthetic micro-organisms, such as some species of *Euglena* and diatoms which may exist heterotrophically, light often leads to the disappearance of PEPCK and a major change in the metabolism of the organism (Mortain-Bertrand *et al*., 1987; Pönsgen-Schmidt *et al*., 1988; Descolas-Gros and Oriol, 1992). The mechanism that brings about these changes in the abundance of PEPCK has been investigated in some bacteria and yeast. In yeast, sugars decrease the activity of PEPCK by inactivation of the enzyme by phosphorylation, increased proteolytic degradation and a repression of transcription of the gene (Proft *et al*., 1995, and references therein). These processes are often studied by identifying the protein that is lacking in mutants of yeast defective in these processes (Proft *et al*., 1995).

3. *Flowering Plants*

In flowering plants the abundance of PEPCK is strictly controlled. PEPCK is only present in certain types of cell, and in many of these only under certain conditions (Edwards *et al*., 1971; Walker *et al*., 1999; Chen *et al*., 2000; Walker *et al*., 2001). The abundance of PEPCK protein is often dependent on developmental stage; for example, it is most abundant in grape seeds at a certain stage of development (Walker *et al*., 1999) and it is present in the flesh of tomato only during ripening, when organic acids may be converted to sugars by gluconeogenesis (Bahrami *et al*., 2001). Similarly, in cotyledons of germinating seeds, in which gluconeogenesis from storage reserves occurs, the abundance of PEPCK changes greatly during development (Leegood and ap Rees, 1978a). In some cases, such as ripening tomato (Bahrami *et al*., 2001) and germinating cucumber seeds, changes in PEPCK protein reflect changes in the abundance of PEPCK mRNA (Kim and Smith, 1994). In addition, the abundance of PEPCK in many tissues is altered by physiological factors; for example, it appears to be increased by treatments that lower intracellular pH (Walker *et al*., 2001). In leaves of C4 plants, in which PEPCK functions in photosynthesis, illumination increases the abundance of PEPCK mRNA; however, the mechanism responsible is unknown (Finnegan *et al*., 1995, Finnegan and Burnell, 1999; Furumoto *et al*., 1999). In marked contrast to vertebrates, almost nothing is known about how the abundance of PEPCK protein in cells of flowering plants is regulated.

B. MECHANISMS THAT BRING ABOUT A RAPID CHANGE IN PEPCK ACTIVITY

The need, at least in some tissues, for PEPCK activity to be modulated in ways other than by changes in the abundance of PEPCK protein is clearly shown in leaves of a number of CAM plants. In these, PEPC is involved in the synthesis of organic acids during the night, and PEPCK in their subsequent decarboxylation during the day. However, both enzymes are present in the cytosol of the same cells at the same time, which suggests that if a futile cycle of carboxylation and decarboxylation is to be avoided then there must be a mechanism that modulates PEPCK activity (Walker, 1992). An important part of this mechanism is that PEPCK from flowering plants is subject to reversible protein phosphorylation (Walker *et al.*, 1995). Interestingly, Utter and Kolenbrander (1972) suggested: 'that alterations in the concentration of OAA may serve as a regulatory device for this enzyme'. In effect this is what phosphorylation does – it alters the catalytic velocity of PEPCK at a given concentration of OAA (Walker *et al.*, 2002). This supports the view that the concentration of OAA is a critical factor in determining the catalytic velocity of PEPCK *in vivo* in all living organisms.

1. Flowering Plants

PEPCK is subject to reversible protein phosphorylation in flowering plants

PEPCK from flowering plants possesses an extension of 12 kDa at its N-terminus, compared with PEPCK–ATP of otherwise similar sequence from other groups of organisms (Kim and Smith, 1994). PEPCK is *in vivo* phosphorylated at a site(s) within the N-terminal extension in germinating seeds, in leaves of CAM and some C4 plants (Walker and Leegood, 1995, 1996). This was shown by feeding tissue ^{32}Pi and immunoprecipitating PEPCK from extracts of the tissue at various times after homogenisation. The loss of the N-terminal extension by proteolysis in these extracts correlated with the loss of ^{32}Pi (Walker and Leegood, 1995, 1996). Similarly, intact PEPCK is phosphorylated by cyclic AMP-dependent protein kinase (cAMP-dependent protein kinase) *in vitro*, whereas the enzyme that lacks the N-terminal extension is not (Walker and Leegood, 1995). Phosphorylation *in vitro* by cAMP-dependent protein kinase brings about the same changes in the catalytic properties of PEPCK as are caused by phosphorylation *in vivo* (Walker *et al.*, 2002). Although PEPCK is phosphorylated in all germinating seeds and leaves of CAM plants studied, in C4 plants the N-terminal extension is usually truncated and often not subject to phosphorylation (Walker and Leegood, 1996; Walker *et al.*, 1997). PEPCK is also phosphorylated in leaves of C3 plants such as cucumber, in which it is located in the trichomes and vasculature, and in roots (Walker and Leegood, 1996; R. P. Walker, unpublished). PEPCK is phosphorylated under conditions – such as in the leaves of C4 grasses during darkness – in which it would be expected that the enzyme is inactive (Walker and Leegood, 1996). The N-terminal extension of PEPCK from many species of flowering plants contains two potential phosphorylation sites; one is a motif for AMP-dependent protein kinase

and the other a motif for cAMP-dependent protein kinase (Walker *et al*. 2002). This site is also a binding site for 14,3,3 proteins. These proteins bind to target proteins when the target is phosphorylated and, in the case of many enzymes, modulates their activity (for review, see Chung *et al*., 1999). The possession of more than one phosphorylation site may be potentially important in allowing different signal transduction pathways to alter the phosphorylation state of PEPCK. Proteolytic digests of pure PEPCK from *P. maximum* leaves, phosphorylated either *in vivo*, or *in vitro* by cAMP-dependent protein kinase, showed a similar peptide pattern after SDS–PAGE using tricine gels. In both cases only the same single peptide was labelled. However, because the two potential phosphorylation sites are close together this does not prove that the same site is phosphorylated *in vitro* and *in vivo*. An antibody was raised against a peptide that corresponded to a small part of the phosphorylated cAMP-dependent protein kinase site on PEPCK. The serum was affinity-purified by incubation with either purified phosphorylated PEPCK or non-phosphorylated PEPCK, and its specificity was checked on blots of SDS–PAGE gels. This antibody was generated, and affinity purification performed, essentially as described for antibodies against amino acids (Walker *et al*., 2001). The antiserum incubated with phosphorylated PEPCK only recognised non-phosphorylated PEPCK and *vice versa*. This may have been because the peptide used as an immunogen was only partially phosphorylated. If leaves of C4 plants were fed ^{32}Pi, and phosphorylated and non-phosphorylated PEPCK purified, changes in the amount of radiolabelling of PEPCK corresponded with changes in cross-reactivity with the antibody that recognised the phosphorylated form of PEPCK (Fig. 2). In addition, if phosphorylation of non-phosphorylated PEPCK was done *in vitro* using cAMP-dependent protein kinase, phosphorylated PEPCK so obtained was recognised by the antiserum (Fig. 2). These results strongly suggest that the site phosphorylated *in vivo* in darkened leaves of *P. maximum* is the cAMP-dependent protein kinase site. The antibody against non-phosphorylated PEPCK specifically recognised only non-phosphorylated PEPCK in crude extracts of *P. maximum* leaves (R. P. Walker unpublished observations). This antibody will be useful in determining the phosphorylation state of PEPCK *in vivo* in a number of plant species under different conditions because feeding ^{32}Pi to tissues, in order to determine the phosphorylation state of an enzyme, usually requires the tissue to be damaged. In contrast to estimating the phosphorylation state of an enzyme by looking at changes in the abundance of mRNA for the relevant protein kinase, the antibody method directly shows the phosphorylation state of the enzyme.

What effect does phosphorylation have on PEPCK in vitro*?*

PEPCK activity can be measured in *in vitro* assays either in the decarboxylation or carboxylation direction; however, unless the enzyme is pure it is difficult to use the decarboxylation assay for detailed studies of its catalytic properties, because malate dehydrogenase, which is very abundant in extracts of plant tissues, will convert the substrate OAA to malate (Hatch and Mau, 1977).

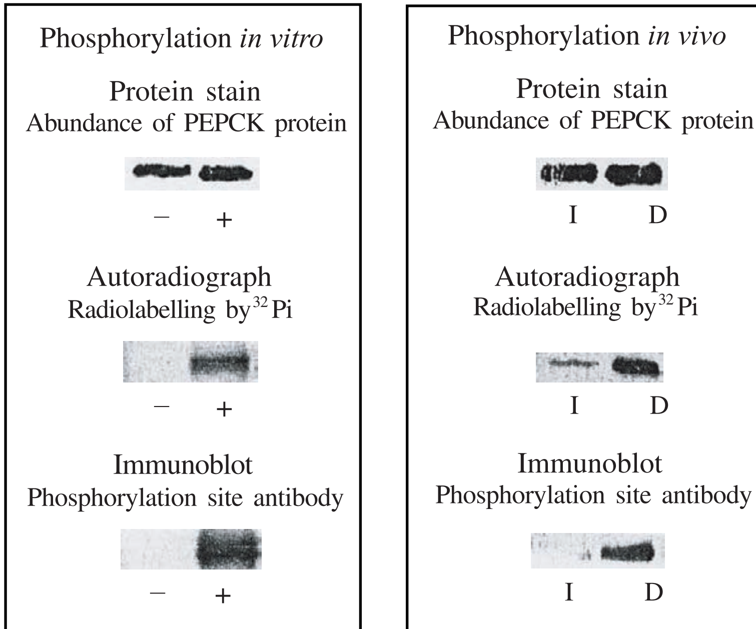


Fig. 2. An antibody raised to the phosphorylated cAMP-dependent protein kinase site, possessed by PEPCK, recognises PEPCK phosphorylated *in vivo* in *P. maximum* leaves and PEPCK from *P. maximum* leaves phosphorylated *in vitro* by cAMP-dependent protein kinase, but not the non-phosphorylated enzyme. Detached leaves of *P. maximum* were fed ^{32}Pi as described by Walker and Leegood (1996). Non-phosphorylated enzyme was purified from illuminated leaves and phosphorylated enzyme from darkened leaves, as described by Walker *et al.* (2002). *In vitro* phosphorylation of non-phosphorylated PEPCK was done using the catalytic subunit of cAMP-dependent protein kinase, as described by Walker and Leegood (1995). Electrophoretic methods were carried out as described by Walker and Leegood (1996). I = PEPCK from illuminated leaves; D = PEPCK from darkened leaves. – = PEPCK from illuminated leaves before phosphorylation by cAMP-dependent protein kinase; + = PEPCK from illuminated leaves after phosphorylation by cAMP-dependent protein kinase.

Measurement of the carboxylase activity of PEPCK in crude extracts of either illuminated or darkened leaves of both CAM or C4 plants showed no change in activity of PEPCK in *in vitro* assay under conditions which measure maximum activity (Carnal *et al.*, 1993; Walker *et al.*, 1997; Walker *et al.*, 2002). Similarly, there is no change in affinity for its substrates when PEPCK activity is measured under these conditions. PEPCK was purified separately from illuminated or darkened leaves of the C4 grass *Panicum maximum* – a tissue in which PEPCK is abundant (Edwards *et al.*, 1971; Hatch and Mau, 1977) and its phosphorylation state easily defined (Walker *et al.*, 2002). Studies using the purified enzyme showed that phosphorylation alters PEPCK activity in the following ways. In the

decarboxylation direction it lowers its affinity for OAA. The difference in activity between phosphorylated and non-phosphorylated enzyme changes from about 10-fold at 5 μM OAA to three-fold at 20 μM OAA, to a 30% difference at 100 μM OAA. This is interesting because the concentration of OAA in the cytosol of many cells is about 5–20 μM (see Titheradge *et al*., 1992; Petersen *et al*., 2001; Agostino *et al*., 1996). In addition, the effect of phosphorylation on the affinity of PEPCK for OAA is modulated by the ratio of ATP to ADP, the difference in activity is greater the higher the ratio of ATP to ADP. For example, at 20 μM OAA and a ATP to ADP ratio of 9:1, there is a three-fold difference in activity, but at a ratio of 1:1 only about a 50% difference. In illuminated C3 leaves the ATP to ADP ratio in cytosol is about six (Gardeström, 1987). In the carboxylation direction activity is also dependent on the concentration of PEP and the ratio of ATP to ADP (Walker *et al*., 2002) and phosphorylation lowers the affinity of the enzyme for PEP. The enzyme from flowering plants that lacks the N-terminal extension, and PEPCK from yeast, behave the same as the non-phosphorylated enzyme. Interestingly, the ratio of ATP to ADP alters the affinity of all these forms of PEPCK for both PEP and OAA several-fold; this is most pronounced at physiological concentrations of OAA or PEP, and phosphorylation just amplifies this response (Walker *et al*., 2002). It therefore seems likely that the effect of the ratio of ATP to ADP on the affinity of PEPCK for PEP and OAA is an inherent property of many ATP-dependent PEPCKs, and that phosphorylation of the N-terminal extension just enhances these properties.

What is known about the kinase that phosphorylates PEPCK?

PEPC-kinase, the protein kinase that is thought to phosphorylate PEPC in at least some tissues *in vivo*, was cloned recently (Hartwell *et al*., 1999). A number of observations raise the possibility that PEPC-kinase may be responsible for the phosphorylation of both PEPC and PEPCK in some tissues. Firstly, partially purified PEPC-kinase from maize phosphorylates PEPCK from cucumber at a site(s) within the N-terminal extension (Walker and Leegood, 1995). Secondly, both PEPC and PEPCK are phosphorylated by cAMP-dependent protein kinase at the same site that is phosphorylated *in vivo*, and for both PEPCK and PEPC, phosphorylation alters the regulatory properties in the same way as phosphorylation *in vivo* (Walker and Leegood, 1995; Chollet *et al*., 1996; Walker *et al*., 2002 and references therein). Thirdly, in leaves of CAM plants, PEPCK and PEPC are both present in the cytosol of the same cells, and changes in their phosphorylation state follow the same pattern (Walker and Leegood, 1996). Phosphorylation activates PEPC (see Chollet *et al*., 1996) and inactivates PEPCK (Walker *et al*., 2002). This is consistent with the requirement that when PEPCK is active PEPC is inactive and *vice versa*. Fourthly, Giglioli-Guivarc'h *et al*. (1996) showed that an increase in cytosolic pH, which occurred upon illumination of mesophyll cells prepared from leaves of the C4 plant *Digitaria sanguinalis*, is likely to be a component of the signal transduction pathway leading to the appearance of PEPC-kinase. When PEPC activity is measured in *in vitro* assay, a lowering of

pH reduces its activity by amplifying the inhibitory effect of malate and reducing the stimulatory effects of phosphorylation and glucose-6-phosphate. A fall in cytosolic pH is thought to be one of the major factors that regulates the *in vivo* activity of PEPC in leaves of *Sorghum vulgare*; a fall in cytosolic pH from 7.2 to 7.1 has been calculated to reduce the activity of PEPC six-fold at saturating light (Echevarria *et al.*, 1994). In contrast, large amounts of PEPCK protein appear in a range of plant tissues in which PEPCK is not normally present, when they are treated in ways that are known to lower cytosolic pH (Walker *et al.*, 2001). Interestingly PEPCK and PEPC may be components of a mechanism that is likely to be of critical importance in pH homeostasis in plants; this is discussed in detail in section IX.F. A rise in pH increases the activity of PEPC *in vivo*, which causes a decrease in pH. A fall in pH increases the activity of PEPCK *in vivo*, which causes a rise in pH. Together these observations raise the possibility that a change in cytosolic pH is an important signal in the coordinated regulation of PEPCK and PEPC activity in plant cells under certain circumstances. It will be interesting to see whether pure PEPC-kinase phosphorylates PEPCK, and whether changes in the abundance of PEPC-kinase *in vivo* are correlated with changes in the phosphorylation state of PEPCK in a range of tissues.

How does phosphorylation alter the catalytic activity of PEPCK in vivo*?*
A critical question is whether the changes in the catalytic properties of PEPCK brought about by phosphorylation are important *in vivo*. Interestingly, Utter and Kolenbrander (1972) suggested: 'that alterations in the concentration of OAA may serve as a regulatory device for this enzyme'. In effect this is what phosphorylation does; it alters the catalytic velocity of PEPCK at a given concentration of OAA. Further, Utter and Kolenbrander (1972) suggested that: 'It is possible that PEPCK activity is also influenced by the relative phosphorylation states of adenine and guanine nucleotides'. This is also an important factor. The physiological relevance of phosphorylation of PEPCK is discussed in the following sections.

Gluconeogenesis in germinating seeds. Many years ago it was suggested that PEPCK is the rate-limiting step in gluconeogenesis in liver under certain circumstances (see Utter and Kolenbrander, 1972). Recent work has supported this, although it must be stressed that this is only under certain circumstances (Titheradge *et al.*, 1992). In germinating marrow it has been proposed that PEPCK is a regulatory enzyme and exerts a high degree of control over flux through gluconeogenesis (Leegood and ap Rees, 1978b; Trevanion *et al.*, 1995a,b). Leegood and ap Rees (1978b) based this proposal on the observation that the reaction catalysed by PEPCK is displaced from equilibrium and on a 'cross-over experiment'. In the cross-over experiment, gluconeogenic flux was altered using the inhibitor of PEPCK, 3-mercaptopicolinic acid. It was reported that when gluconeogenic flux increased, OAA content decreased and PEP content increased. The problems with this approach are that it is extremely difficult to measure the intracellular concen-

tration of OAA and PEP (for discussion see Petersen *et al*., 2001), and to know the correct value for the equilibrium constant (to measure the equilibrium constant, PEPCK is incubated with substrates *in vitro* and the concentration of substrates and products at equilibrium measured) (Wood *et al*., 1966). The final concentrations depend on the rates of the carboxylation and decarboxylation reactions, which are very dependent on a number of factors such as metal ion concentration and ratio of A(G)TP to A(G)DP (see Chen *et al*., 2002; Walker *et al*., 2002). According to the cross-over theory, an increase in flux through a reaction *in vivo*, and an associated decrease in concentration of the substrate of the enzyme *in vivo*, provide evidence that the enzyme is regulatory (for a discussion of these ideas, see Newsholme and Start, 1973). In the case of PEPCK, this implies that even though the concentration of the substrate OAA decreases *in vivo*, the flux through the PEPCK reaction increases. This would require the catalytic velocity of PEPCK to increase, even though the concentration of its substrate OAA was less. Utter and Kolenbrander (1972) suggested that changes in the concentration of OAA may serve to alter flux through the PEPCK reaction *in vivo*. The experiments of Petersen *et al*. (2001) support this; it was shown that in the bacterium *Corynebacterium glutamicum*, the flux through the PEPCK reaction was proportional to OAA concentration *in vivo*. This suggests that for the observations of Leegood and ap Rees (1978b) to be correct, the enzyme from flowering plants must possess the ability to increase its catalytic rate, independent of OAA concentration. Recent work strongly supports this – PEPCK from flowering plants, unlike that from *C. glutamicum*, is subject to reversible phosphorylation, and the effect of dephosphorylation of PEPCK is to increase its catalytic rate about four-fold at a physiological concentration of OAA (about 10–25 μM), and a physiological ATP to ADP ratio of 6 (see fig. 3 in Walker *et al*., 2002). In germinating cucumber cotyledons, PEPCK is dephosphorylated in response to light (Walker and Leegood, 1995), which is known to increase the rate of lipid mobilisation in this seed (Davies *et al*., 1981). This is probably because when the seed germinates in the soil it then needs to conserve its storage reserves (many of which are used in the assembly of the photosynthetic apparatus) upon reaching light.

C4 photosynthesis. An interesting example of the importance of OAA concentration in relation to catalytic activity *in vivo* is provided by C4 photosynthesis, which is an example of a photosynthetic CO_2-concentrating mechanism. In C4 photosynthesis CO_2 is fixed into dicarboxylic acids by the action of PEPC in the mesophyll cells of the leaf. The malate and/or amino acids so produced diffuse into a layer of cells underlying the mesophyll, called the bundle sheath. In these cells, decarboxylation occurs, CO_2 enters the Benson–Calvin cycle, and sugars are produced (for a review, see Hatch, 1987). In many species PEPCK is used to decarboxylate organic acids in the bundle sheath.

The maximum catalytic activity of PEPCK in extracts of germinating marrow seeds is about 7.0 units of PEPCK per gram fresh weight (Leegood and ap Rees, 1978a) (one unit = 1 μmol min^{-1} of substrate converted to product at

25°C and under other conditions in *in vitro* assay in which maximum activity is measured). This is 70-fold (Trevanion *et al.*, 1995a) higher than the gluconeogenic flux. In rat liver, which contains similar amounts of PEPCK, the rate of gluconeogenesis from lactate is about seven times less than the content of PEPCK (cf. tables 6.6 and 6.9 in Newsholme and Start, 1973). This discrepancy can be explained, for plants at least, by a comparison of the catalytic properties of the enzyme and the concentration of OAA *in vivo*. If we assume the ATP:ADP ratio in the cytosol of germinating seeds is between three and eight, as it is in many organisms (Newsholme and Start, 1973; Gardeström, 1987) and the concentration of OAA is about 10–20 μM, as is common in other cells (Titheradge *et al.*, 1992; Agostino *et al.*, 1996; Petersen *et al.*, 2001), then flux through PEPCK would be between about 2 and 20% of its maximum catalytic activity (see fig. 3 in Walker *et al.*, 2002). However, the rate of photosynthesis in leaves of C4 plants, and hence flux through the PEPCK reaction, is up to about 5.0 μmol min^{-1} g (fresh $wt)^{-1}$ (Burnell and Hatch, 1988b). To increase flux through the PEPCK reaction, the abundance of PEPCK could be increased; however, the maximum catalytic activities of PEPCK in extracts of germinating seeds and leaves of PEPCK/NAD–malic enzyme C4 plants are similar (Walker *et al.*, 1995, 2002). The second alternative is to increase the concentration of OAA. The concentration of OAA in these cells *in vivo* is thought to be about 100–150 μM (Agostino *et al.*, 1996). In different species of C4 plants, either malate or aspartate is imported into the bundle sheath cells. The C4 plants that use PEPCK to decarboxylate OAA import large amounts of aspartate into the bundle sheath (Chapman and Hatch, 1981; Burnell and Hatch, 1988b). There are two subgroups of C4 plants that utilise PEPCK as a decarboxylase, one uses PEPCK in conjunction with mitochondrial NAD–malic enzyme (Burnell *et al.*, 1988b). The other type, an example being maize, uses PEPCK in conjunction with chloroplastic NADP–malic enzyme (Walker *et al.*, 1997). Both contain large amounts of aspartate aminotransferase in the bundle sheath. This allows a high concentration of OAA to be achieved in the cytosol of the bundle sheath. The reason for this is that the equilibrium constant of the reaction catalysed by aspartate aminotransferase favours the formation of a high concentration of OAA, whereas that of the malate dehydrogenase reaction results in a much lower concentration of OAA. In the presence of this higher concentration of OAA and an ATP to ADP ratio of three to seven, which is typical of photosynthetic cells of C3 plants (Gardeström, 1987), PEPCK would be operating at about 50% of its maximum catalytic velocity (see fig. 3 in Walker *et al.*, 2002). PEPCK/NAD–malic enzyme C4 plants contain between about seven and nine units of PEPCK per gram fresh weight (Hatch and Mau, 1977; Smith and Woolhouse, 1983; Burnell, 1986; Walker *et al.*, 2002), when PEPCK activity is measured in *in vitro* assay under conditions which measure its maximum activity. Therefore, the amount of *in vivo* activity would be sufficient to just support the maximum rate of photosynthesis in PEPCK/NAD–malic enzyme C4 plants.

It should be noted that NAD–malic enzyme also supports photosynthesis in these plants – the contribution being about 25% of that of PEPCK (Burnell and Hatch, 1988b).

The question arises as to how the activity of PEPCK is coordinated with that of photosynthesis, for example, how is PEPCK inactivated in the dark? One possibility, as in gluconeogenesis in vertebrates and germinating seeds, is that the abundance of PEPCK is altered. However, this does not appear to be the case (Hatch and Mau, 1977; Carnal *et al.*, 1993; Walker *et al.*, 2002). Also, despite extensive study for nearly 50 years, there is no evidence in any organisms that any metabolite, or combination of metabolites, could sufficiently decrease flux through the PEPCK reaction when OAA is at a concentration of 100–150 μM. Furthermore, there is no evidence of any other modifications to the protein other than reversible protein phosphorylation which could inactivate PEPCK. At high concentrations of OAA, phosphorylation would be insufficient to inactivate PEPCK (see fig. 3 in Walker *et al.*, 2002). In addition, the N-terminal extension is truncated, lacks the phosphorylation site recognised by cAMP-dependent protein kinase, and is not phosphorylated *in vivo* in leaves of many species of C4 plants (Walker and Leegood, 1996; Walker *et al.*, 2002). In contrast, the N-terminal extension is not truncated, and the enzyme is subject to phosphorylation in a range of germinating seeds and leaves of CAM plants (Walker and Leegood, 1995, 1996; Walker and Leegood 1997). This shows that phosphorylation is not essential for regulation of PEPCK activity in the leaves of at least some species of C4 plant. In the C4 plant *U. panicoides* there is a small multigene family for PEPCK, and it is possible that this may allow forms that are not subject to phosphorylation to be expressed in leaves, and forms that are subject to phosphorylation to be expressed in other tissues, although this is uncertain (Finnegan *et al.*, 1999).

To understand how flux through the PEPCK reaction is turned off in the dark, it is important to consider the size and origin of the OAA pool and the catalytic and regulatory properties of PEPCK. This is because the only known mechanism by which flux through the PEPCK reaction could be greatly reduced would be a decrease in the concentration of OAA. Firstly, to consider the size and origin of the OAA pool; if OAA is at a concentration of 150 μM in the cytosol, and the cytosol is about 10% of cell volume, then in one gram fresh weight of bundle sheath there are about 15 nmol of OAA. This would mean that PEPCK in this tissue, operating at 25% of its maximum rate, would consume this pool in one-third of a second. The next step is to consider the origin of OAA. OAA arises from aspartate by the action of aspartate aminotransferase:

$$\text{aspartate} + \alpha\text{-ketoglutarate} \leftrightarrow \text{glutamate} + \text{OAA}$$

The equilibrium constant of the reaction is given by [glutamate][OAA]/[aspartate][α-ketoglutarate] and is approximately one (Mavrovouniotis, 1991). Next, it is important to have an idea of the concentration of these metabolites.

The concentration of OAA in these cells *in vivo* is thought to be about 100–150 μM (Agostino *et al*., 1996). The mesophyll cells, in which organic acids are synthesised, and the bundle sheath cells, in which decarboxylation occurs, are connected together by numerous plasmodesmata. Plasmodesmata are somewhat like pores between plant cells – there is no plasma membrane between the cells, and the cytosol of the two cells is in direct connection. In leaves of C4 plants this allows metabolites to diffuse between the cells according to the concentration gradient (for a review, see Hatch, 1987). A surprising fact is that if bundle sheaths, or their cells, are isolated from leaves, then these plasmodesmata remain open. This is a very useful experimental system because if these isolated tissues are illuminated in the presence of appropriate compounds then photosynthesis occurs to a similar extent to intact leaves (Burnell and Hatch, 1988a). The presence and abundance of different compounds in the bathing medium greatly alters the rate of photosynthesis. For example, inclusion of the inhibitor of PEPCK, 3-mercaptopicolinic acid, or the omission of Mn^{2+}, stops photosynthesis under certain conditions (Burnell and Hatch, 1988a). This allows the effects of potential inhibitors of PEPCK, for example, to be studied *in vivo* (Carnal *et al*., 1993). The isolated bundle sheath system has made invaluable contributions to the understanding of C4 photosynthesis. In the present discussion it allows us to know what concentrations of apartate and α-ketoglutarate are necessary to generate sufficient OAA for the PEPCK reaction *in vivo*. Burnell and Hatch (1988b) found that by incubating bundle sheath cells in the presence of different concentrations of aspartate, a concentration of about 2 mM was sufficient for the PEPCK reaction. A similar concentration was required for α-ketoglutarate (see fig. 3 in Burnell and Hatch 1988b). Glutamate, a product of aspartate aminotransferase, did not inhibit the PEPCK reaction. These values are not too different from studies in which these metabolites were measured in extracts of maize leaves; in addition, the concentration of glutamate was measured in these extracts (Leegood, 1985; Stitt and Heldt, 1985). If it is assumed that glutamate is 40 mM, aspartate 3 mM, α-ketoglutarate 2 mM and OAA 150 μM, then the reaction catalysed would be in equilibrium ($[40 \times 0.15]/[3 \times 2] = 1$; where 1 is the equilibrium constant for the reaction). The exact values of these concentrations are not so important – similar results would be obtained if the values were substantially different, i.e. the calculation just illustrates a point. If the concentration of OAA falls to 10 μM then the concentration of the other components of the equilibrium will change; for example 42 mM glutamate, 0.65 mM aspartate and 0.65 mM α-ketoglutarate would be in equilibrium. The change in the sum of the concentration of the reactants of aspartate aminotransferase would be very small, from 45 mM at 150 μM OAA to 43.3 mM at 10 μM. If the cytosol occupies 10% of cell volume then 0.17 μmol of reactant are lost; this would occur in seconds. It should be noted that in the natural situation the increase in illumination as the sun rises, for example, occurs over a considerable period of time, and that a gradual change in the concentration of these metabolites would occur. The point of this simplified (the system has been

viewed as closed) and approximate calculation is to show that it is possible to readjust the concentration of OAA without radically altering the pools of other metabolites within the cell. It should be noted that it is reported not to be possible to reliably measure OAA in extracts of tissues, because of its extreme instability (Rej, 1985; Agostino *et al*., 1996; Petersen *et al*., 2001).

Carnal *et al*. (1993) discussed the possibility that in the dark, the reaction catalysed by PEPCK approached equilibrium, so that there was no net flux. In keeping with the known catalytic properties of PEPCK, they came to the only reasonable conclusion, that this was unlikely. However, our understanding of the catalytic properties of PEPCK has increased in recent years, not least because we now know that – at least for the enzyme from flowering plants – it possesses a high affinity for CO_2 under physiological conditions (Chen *et al*., 2002). We propose that in leaves of C4 plants in the dark, OAA concentration decreases greatly, that the rates of the decarboxylase and carboxylase reactions become similar, and that there is a minimal net flux. We will now explore this proposal a little more. Firstly, we will consider the effects of the ATP to ADP ratio; in illuminated leaves of C3 plants the adenylate ratio increases from about three in the dark to seven in the light (Gardeström, 1987). It is known that ADP inhibits the decarboxylase activity *in vitro* of PEPCK from C4 plants (Burnell, 1986); similar results have been obtained for PEPCK from other living things. Burnell (1986) suggested that this inhibition might have a role *in vivo*. *In vivo*, the formation of PEP by bundle sheaths in the presence of 1 mM ATP is inhibited about 30–40% by the addition of 1 mM ADP (Carnal *et al*., 1993). A similar amount of inhibition is seen in *in vitro* assay (see fig. 3 in Walker *et al*., 2002). Secondly, we need to consider the effect of other possible effectors. Hatch and Mau (1977) and Burnell (1986) found that a number of sugar phosphates inhibited PEPCK in *in vitro* assay by about 60% at a concentration of 5 mM. However, detailed investigation showed that this effect was complex because a number of interactions occurred (Burnell, 1986). The inhibition could be alleviated by increasing the concentration of Mn^{2+} (fig. 6 in Burnell, 1986). Mg^{2+} normally inhibits PEPCK from flowering plants by about 50% this being a very complex effect (Burnell, 1986; Chen *et al*., 2002). However, if both sugar phosphates and Mg^{2+} are present, inhibition is never more than 60%, that is, their effects are not additive (see fig. 6 in Burnell, 1986). Therefore, under physiological conditions, that is in the presence of Mg^{2+}, sugar phosphates are not inhibitors. This is supported by *in vivo* bundle sheath assays in which these compounds did not inhibit the activity of PEPCK (Carnal *et al*., 1993). An interesting observation was that the addition of 1 mM PEP to bundle sheath assays inhibited PEPCK activity by about 30–40% (Carnal *et al*., 1993). Whether this is the case *in vitro* would be interesting to determine. It would also be interesting to determine whether OAA inhibits the carboxylase activity.

If the rates of the carboxylase and decarboxylase activity of PEPCK are similar in darkened leaves of C4 plants, the ATP to ADP ratio is about three, and the OAA concentration is 10 μM – this would require a PEP concentration of

about 1 mM (see fig. 3 in Walker *et al.*, 2002), a value similar to that reported in maize (Leegood, 1985; Stitt and Heldt, 1985). There are likely to be two related reasons why regulation of PEPCK by phosphorylation is not essential in leaves of C4 plants. Firstly, at a high concentration of OAA, the effect of phosphorylation on PEPCK activity is much smaller than at a low concentration of OAA (see fig. 3 in Walker *et al.*, 2002). Secondly, if the reaction is operating in both directions in the dark, phosphorylation would have little or no effect on net flux (see fig. 3 in Walker *et al.*, 2002). It is possible that an interesting extension of this mechanism of control occurs in photosynthetic CO_2-concentrating mechanisms in some algae, in which it is possible that PEPCK acts as both a carboxylase and decarboxylase. This is considered in detail in section IX.C.6. A more extreme case still may be seen in organisms which use PEPCK as a carboxylase – in these it is likely that a very low OAA concentration must be maintained. It is unfortunately beyond the scope of this article to describe how the C4 cycle as a whole is coordinately regulated, but some aspects of this complex topic are considered by Burnell and Hatch (1988b), Furbank *et al.* (1991), Carnal *et al.* (1993), Agostino *et al.* (1996) and Leegood and Walker (1999).

CAM. Crassulacean acid metabolism (CAM) is another example of a mechanism that assists plants in acquiring CO_2 for photosynthesis. In these plants, PEPCK is likely to be regulated in a different way to the C4 plants. Many familiar plants, such as cacti (although these do not utilise PEPCK) and bromeliads (many of which are grown in the home and do utilise PEPCK) are CAM plants. In the leaves of many CAM plants the stomata open at night, and CO_2 enters the leaf to be fixed into organic acids by PEPC. These organic acids, usually malic acid or citric acid, are accumulated in the vacuole. During the day the stomata close, organic acids leave the vacuole, are decarboxylated, and the CO_2 thus released enters the Benson–Calvin cycle. This mechanism reduces water loss because more water vapour passes through the stomata during the day when temperatures are higher (Ting, 1985). CAM may be an elaboration of a mechanism in which, during drought, plants close their stomata and use decarboxylation of their organic acid reserves to provide CO_2 for photosynthesis. In CAM plants, either PEPCK or the malic enzymes may be used to decarboxylate organic acids – this depends on the species (Dittrich *et al.*, 1973). An interesting observation is that in species that use PEPCK as a decarboxylase, its abundance, as indicated by its maximum activity in *in vitro* assay, is often reported to be higher than that of the malic enzymes in species which use these as a decarboxylase (see Christopher and Holtum, 1996). In CAM plants the activity of PEPCK is high; a reworking of the data for pineapple show that it is about 12–18 units per gram fresh weight (Dittrich *et al.*, 1973; Black *et al.*, 1996; Christopher and Holtum, 1996). In *Clusia minor* it is four units per gram fresh weight (Borland *et al.*, 1998), an activity which is about four times in excess of the requirements for its activity *in vivo* (compare Borland and Griffiths, 1997; Borland *et al.*, 1998). It should be noted that expressing PEPCK activity and the rate of photosynthesis on a per gram fresh weight basis is not wholly satisfactory

for CAM plants because they are succulent, and internal tissues are often not photosynthetic and do not contain PEPCK. In CAM plants OAA is derived from malate by the action of malate dehydrogenase, and the concentration of OAA is likely to be lower than in the leaf of a C4 plant. For example, at a concentration of 10–20 μM, and at an ATP to ADP ratio of between three and seven, the catalytic velocity of PEPCK would be between about 10 and 30% of its maximum. This is in keeping with the amounts of PEPCK in leaves of CAM plants, which are between about three and ten times the rate of photosynthesis.

In leaves of many CAM plants, PEPC is involved in the synthesis of organic acids during the night, and PEPCK in their subsequent decarboxylation during the day. However, both enzymes are present in the cytosol of the same cells at the same time, which suggests that if a futile cycle of carboxylation and decarboxylation is to be avoided there must be a mechanism that modulates PEPCK activity (Walker, 1992). The regulatory properties of PEPC were considerably simpler to find than those for PEPCK, and in the 1960s the effectors of the enzyme were found (see Utter and Kolenbrander 1972; Latzko and Kelly, 1983). In the 1980s it was found that the sensitivity of the enzyme to these effectors was modulated by reversible protein phosphorylation which activates the enzyme (see Chollet, 1996). These effects are thought to reduce the velocity of the PEPC reaction during the day in leaves of CAM plants. PEPCK is subject to reversible protein phosphorylation in leaves of CAM plants in which it is phosphorylated at night (Walker and Leegood, 1996). At concentrations of 10–20 μM OAA and ATP/ADP ratios of three to seven, which are typical of many types of cell, phosphorylation alters the reaction velocity of PEPCK between three- and ten-fold. This is similar to the amount of change in reaction velocity proposed for PEPC. From dusk to dawn in the natural environment, the ratio of activity of PEPCK to that of PEPC will gradually change. There is likely always to be a substrate cycle between PEPC and PEPCK, similar to that in the bacterium *Corynebacterium glutamicum* in which, under certain conditions, about two-thirds of the OAA synthesised by PEPC are recycled by PEPCK (Petersen *et al.*, 2000). In *C. glutamicum*, this substrate cycle may serve to regulate flux between PEP and OAA and to maintain the intracellular concentrations of OAA and PEP (Petersen *et al.*, 2001). In CAM plants the net flux will vary according to the time of day, and the substrate cycle will serve to amplify the regulatory mechanism. For a discussion of substrate (futile) cycles and their role in regulation, the reader is referred to Newsholme and Start (1973). Substrate cycles can produce a very large change in the amount and direction of net flux through a reaction because flux through one enzyme can be increased and that through the other decreased. An additional factor is likely to enhance this regulatory mechanism further. Malate has been widely believed to be important in the regulation of PEPC *in vivo* for many years. Malate is an inhibitor of PEPC and inhibition is relieved by phosphorylation of the enzyme (Chollet *et al.*, 1996). An increase in the cytosolic concentration of malate is proposed to be a major component of the mechanism that inhibits PEPC during the day in CAM plants

(Chollet *et al*., 1996; Borland *et al*., 1999). However, an increase in malate is likely to increase flux through PEPCK. This is because an increase in malate will increase the concentration of OAA at a given ratio of NAD/NADH (this is because of the equilibrium position of the malate dehydrogenase reaction [malate] × [NAD] = constant × [OAA] × [NADH]). In CAM plants it is important that accurate measurements of the cytosolic concentration of a number of metabolites at different times of the day are obtained. This applies to the regulation of both PEPCK and PEPC. A potentially important further factor in regulation of PEPCK/PEPC are small changes in cytosolic pH. For example, in the *S. vulgare* leaf, a fall in cytosolic pH from 7.2 to 7.1 has been calculated to reduce the activity of PEPC six-fold at saturating light (Echevarria *et al*., 1994). In photosynthetic cells it is thought that the pH of the cytosol is different in illuminated and darkened leaves (see Kurkdjian and Guern, 1989; Raghavendra *et al*., 1993). Recent studies using mesophyll protoplasts prepared from leaves of the CAM plant *Kalanchoe daigremontiana* by Hafke *et al.* (2001) strongly suggest that in the day the pH of the cytosol decreases by 0.3 pH units and that the concentration of malic acid in the cytosol increases by about 10 mM. This would greatly inhibit PEPC activity. In addition, it is possible that PEPC kinase is responsible for phosphorylation of both PEPC and PEPCK in this tissue, and that changes in intracellular pH are a component of the signal transduction pathway, leading to changes in the abundance of mRNA for this kinase. Changes in cytosolic pH are an important second messenger in a number of systems (see Kurkdjian and Guern, 1989; Sakano, 2001).

2. Vertebrates

In mammals there are two forms of PEPCK, one of which is located in the mitochondria and the other in the cytosol, and it is possible that any mechanisms used to rapidly modulate their activity might differ. The most important mechanism that sets the level of activity of cytosolic PEPCK in mammalian tissues is changes in the abundance of the protein (see Hanson and Reshef, 1997). However, other mechanisms may bring about a more rapid change in its activity. Under some circumstances a change in concentration of OAA and/or an alteration of the ratio of ATP to ADP might be important in changing flux through PEPCK (Utter and Kolenbrander, 1972). A range of metabolites were found not to act as effectors of the enzyme (Utter and Kolenbrander, 1972), although more recently α-ketoglutarate has been reported to act as a competitive inhibitor (Titheradge *et al*., 1992).

PEPCK from all organisms requires two cations, millimolar concentrations of one being required because a metal ion–nucleotide is the substrate. This ion is normally Mg^{2+} *in vivo*. Micromolar concentrations of a second cation, thought to be Mn^{2+} *in vivo*, are required to bind within the active site, and this ion plays a role in the catalytic mechanism of the enzyme (for a review, see Matte *et al*., 1997). Nowak *et al.* (1986) and Schramm *et al.* (1986) discuss the possibility

that changes in the Mn^{2+} concentration could act to regulate the activity of mitochondrial and cytosolic PEPCK. This could be achieved by the release of Mn^{2+} from intracellular compartments, similar to the well-known release of Ca^{2+}. Maggini *et al.* (1993) suggested that this was unlikely, at least for the cytosolic enzyme, because of its insensitivity to physiological changes in Mn^{2+} concentrations in an assay which simulated physiological conditions. Whether regulation of PEPCK activity occurs *in vivo* by changes in Mn^{2+} (or other metal ions) concentration remains unknown.

The catalytic activity of PEPCK *in vitro* is very susceptible to loss of activity, which often results from oxidation of –SH groups. Activity can be restored *in vitro* by certain treatments. It has been suggested that certain proteins, such as ferroactivators, permit Fe^{2+} ions to activate cytosolic PEPCK, possibly by maintaining critical cysteine residues at the active site in a reduced state *in vivo* (Bentle *et al.*, 1976; Bentle and Lardy, 1977). Subsequent studies were performed in which a number of ferroactivators were purified and identified, for example, catalase and phosphoglycerate mutase were identified as ferroactivators (see Hanson and Patel, 1994). The physiological importance of ferroactivators, other proteins, or another unknown mechanism that brings about changes in the oxidation state of cysteine residues *in vivo,* remains unknown.

Titheradge *et al.* (1992) proposed a mechanism for the regulation of cytosolic PEPCK under certain conditions. When liver is exposed to glucagon, the gluconeogenic flux increases; however, the concentration of OAA in the cytosol is maintained at about 15 μM and the concentration of α-ketoglutarate decreases (Titheradge *et al.*, 1992 and references therein). From a number of detailed studies, Titheradge *et al.* (1992) established that α-ketoglutarate is an inhibitor of PEPCK *in vitro,* and that the decrease in its concentration *in vivo* is sufficient to account for the increased flux through the PEPCK reaction *in vivo* without a change in OAA concentration.

In general, mitochondrial PEPCK is always present in some tissues (Hanson and Reshef, 1997; Modaressi *et al.*, 1998). This raises the problem of how its activity is coordinated with other metabolic processes. Modaressi *et al.* (1998) suggested that PEPCK might even act as a carboxylase in mitochondria and serve an anaplerotic role. This would most likely require a low concentration of OAA in the mitochondria in order to decrease the decarboxylase activity of PEPCK. In the mitochondria, OAA concentrations are proposed to be lower than in the cytosol, partly because of the higher ratio of NADH/NAD (see Newsholme and Start, 1973). In this context, it will be interesting to see whether mitochondrial PEPCK possesses a higher affinity for CO_2 than is generally thought, as was recently proposed for the enzyme from flowering plants (Chen *et al.*, 2002). The balance between the carboxylase and decarboxylase activities of PEPCK in mitochondria could then be potentially important in the regulation of its activity.

3. Micro-organisms

Nuclear magnetic resonance (NMR) techniques, in combination with strains of *Corynebacterium glutamicum* with an altered abundance of PEPCK, have shown that flux through the PEPCK reaction *in vivo* under certain conditions is dependent solely on the concentration of OAA (Petersen *et al*., 2001). This does not rule out the possibility that, under other conditions, changes in the ratio of ATP to ADP play a role. The purified enzyme had been found to be reponsive to this factor *in vitro* (Jetten and Sinskey, 1993). In *C. glutamicum*, overall flux between PEP and OAA was largely determined by a substrate cycle between PEPCK and PEPC. Similarly, NMR has shown that in *E. coli* and *Bacillus subtilis* a futile ATP-dissipating cycle occurs between PEPC and PEPCK, the magnitude of which depends on the growth conditions (Sauer *et al*., 1999). PEPCK from *E. coli* is reported to be stimulated by millimolar concentrations of Ca^{2+}; however, the physiological significance of this is uncertain (Medina *et al*., 1990).

C. CONCLUDING COMMENTS ON REGULATION

In flowering plants and vertebrates it was generally thought that the reason PEPCK usually acted as a decarboxylase *in vivo* was because its affinity for CO_2 was very low compared with the concentration of CO_2 within the cell. However, PEPCK from flowering plants possesses a high affinity for CO_2 when assayed *in vitro* under conditions that more closely approximate those found within the cell (Chen *et al*., 2002). This then raises the question as to what determines whether PEPCK in flowering plants (and potentially in other organisms), acts as a carboxylase or decarboxylase *in vivo*. The answer is likely to be that at a physiological ratio of ATP to ADP (4:1) the carboxylase activity of PEPCK–ATP is massively inhibited (see fig. 6 in Urbina, 1987; fig. 3 in Walker *et al*., 2002). On the other hand, if the ratio of ATP to ADP is reversed (1:4), then the decarboxylase activity in comparison is much less inhibited (fig. 3 in Walker *et al*., 2002). PEPCK–GTP is also inhibited by ATP (Rohrer *et al*., 1986). It is possible that PEPCK from many living things shows this response to the ratio of ATP to ADP under assay conditions that more closely approximate those within the cell. This then would be an intrinsic property of many PEPCKs that is essential in biasing the poise of the reaction in favour of decarboxylation. It will be interesting to see in *in vitro* assay, under conditions that more closely approximate those found within the cell, whether PEPCK from organisms in which it acts as a carboxylase *in vivo*, show a lower catalytic velocity in the decarboxylase direction at physiological concentrations of OAA and less inhibition of the carboxylase activity by ATP.

In all living organisms it is likely that the concentration of OAA *in vivo* is of critical importance in determining the direction and magnitude of flux through the PEPCK reaction. This is because at physiological concentrations of OAA a small change in its concentration brings about a very large change in the catalytic velocity of the decarboxylase activity of PEPCK (see fig. 3 in Walker

et al., 2002). PEPCK from flowering plants is subject to reversible protein phosphorylation, the effect of which is to alter the catalytic velocity several-fold at a given physiological concentration of OAA. Titheradge *et al*. (1992) proposed that physiological changes in the concentration of α-ketoglutarate brought about a 30% change in flux through the decarboxylase reaction of PEPCK at a fixed concentration of OAA, in liver, under certain circumstances.

Although phosphorylation is one mechanism used by flowering plants to adjust flux through the PEPCK reaction, other mechanisms are also used. One is a substrate cycle involving PEPC, which occurs, for example, in leaves of CAM plants and some bacteria. In the case of CAM plants, both phosphorylation and the substrate cycle contribute to determining the direction and magnitude of flux between OAA and PEP. It is possible that in mammals a substrate cycle involving PEPCK, pyruvate kinase and pyruvate carboxylase serves to alter the direction of net flux between PEP and OAA (Titheradge *et al*., 1992, and references therein). Another possible substrate cycle is that between the carboxylase and decarboxylase activities of PEPCK itself. This would allow rapid changes in the direction and magnitude of flux through PEPCK, and does not consume ATP (unlike a substrate cycle with PEPC), and this could be important in some organisms. The direction and magnitude of net flux out of this cycle is likely to be very dependent on the concentration of OAA and the ratio of ATP to ADP. This cycle may occur in leaves of some C4 plants and a number of photosynthetic CO_2-concentrating mechanisms found in algae. This is discussed in detail in section IX.C.3. Modaressi *et al*. (1998) suggested that PEPCK might act as a carboxylase in vertebrate mitochondria in certain situations. It is possible that a substrate cycle between the carboxylase and decarboxylase activities of PEPCK plays a key role in regulating the direction and magnitude of flux through the PEPCK reaction under certain circumstances in vertebrates. The N-terminal extension that is possessed by the enzyme from flowering plants is not essential for catalysis (Walker *et al*., 1995), and appears to be homologous to the N-terminal region of PEPCK–GTP. It would be interesting to see whether this part of the protein confers different or regulatory properties on other PEPCKs.

IX. PHYSIOLOGICAL ROLE OF PEPCK

PEPCK is present in plants, animals, fungi, protoctists and bacteria. In unicellular organisms its abundance is greatly changed by environmental factors, and in multicellular organisms PEPCK is localised in certain tissues in which its abundance often changes in response to both the development and the physiological status of the organism. The abundance of PEPCK is often high, between about one part in 100 and one part in 300 of total protein. PEPCK is involved in a number of different metabolic processes for several reasons. Firstly, PEP and OAA occupy a central position in metabolism, and the partitioning of carbon skeletons between them by the action of PEPCK is potentially important in the interconversion of sugars, organic acids, amino acids, aromatic compounds and

lipids. Secondly, in some situations, such as anaerobiosis, PEPCK may act as a carboxylase and both synthesise ATP and provide OAA for fermentation reactions. Thirdly, the provision of CO_2 by the decarboxylase activity of PEPCK for photosynthesis is important in some organisms. Fourthly, the conversion of OAA to pyruvate, by PEPCK and pyruvate kinase, consumes a proton. The synthesis of pyruvate from glucose by glycolysis produces a proton. This is utilised in pH homeostasis in some situations. OAA arises from malate or amino acids imported into the tissue, and the formation of pyruvate from these as opposed to glucose is likely to be used to counteract decreases in intracellular pH. In some situations PEP produced by PEPCK may be utilised in gluconeogenesis, which is also a proton-consuming process.

In multicellular organisms PEPCK is present in a number of cell types, in many of these – in both vertebrates (Hanson and Patel, 1994; Hanson and Reshef, 1997 and references therein) and flowering plants (Walker *et al*., 1999; Chen *et al*., 2000; Walker *et al*., 2001) – its function is uncertain. In most types of cell in multicellular organisms, the pathways used by central metabolism and the amount of flux through each branch are uncertain. This is partly a result of difficulties in studying cell-specific metabolism unless a cell type is highly enriched in certain tissues or large amount of the cell type can be purified, and because of the considerable amount of work needed to perform detailed investigations of a large number of different types of cell. A further complexity is that it is likely that the pathways used differ between species, stage of development and physiological condition of the organism. However, it is possible by considering the diverse functions of PEPCK in living organisms that information obtained studying one group of organisms might provide valuable clues to its function in another.

A. GLUCONEOGENESIS

1. Vertebrates

The role of PEPCK in gluconeogenesis in vertebrates has been discussed in detail. Therefore this topic will be considered only briefly and the reader is referred to Utter and Kolenbrander (1972), Hanson and Mehlman (1976) and Hanson and Patel (1994). Following the discovery of PEPCK in liver (Utter and Kurahashi, 1953) a number of studies in the 1960s and early 1970s established that its function was in gluconeogenesis. Firstly, PEPCK is present in large amounts in tissues in which gluconeogenesis occurs and it is absent or at low abundance in many non-gluconeogenic tissues. Secondly, the abundance of the enzyme changes greatly in response to dietary or hormonal manipulation, and in these its abundance is correlated with the magnitude of the gluconeogenic flux. Thirdly, studies with microbial systems showed that mutants lacking PEPCK could not grow on gluconeogenic substrates, but could if glucose was present (Utter and Kolenbrander, 1972 and references therein).

In vertebrates, the liver and kidney are the main sites of gluconeogenesis. However, the amount of gluconeogenesis that occurs in the kidney is dependent on the species. In the kidney, gluconeogenesis occurs in response to acidosis and can also be viewed as a mechanism used to consume protons arising from the export of NH_3 into the urine. In the urine, NH_3 buffers protons during acidosis. NH_4^+ is produced from the deamination of glutamine and glutamate in the cells of the proximal tubule; NH_3 then diffuses through the plasma membrane into the urine, leaving behind protons in the proximal cell, which must be removed (Ganong, 1977; Tanner, 1978; Silbernagl and Scheller, 1986). Proton consumption is achieved by subsequent metabolism of the α-ketoglutarate arising from deamination reactions. The α-ketoglutarate can either be used in gluconeogenesis or the OAA produced from it can be converted to pyruvate which is utilised by the Krebs cycle; both alternatives utilise PEPCK and are proton-consuming processes. The alternative used depends on the species (Hanson and Patel, 1994). The reason why these processes consume protons is discussed in detail in section IX.F.

Lactate is produced continuously by some cells, such as red blood cells in humans, and intermittently in large amounts by muscle during exercise. Mitochondrial PEPCK is important in the conversion of lactate to sugars. The reason that mitochondrial and not cytosolic PEPCK is often used in gluconeogenesis from lactate is because the conversion of lactate to pyruvate in the cytosol by lactate dehydrogenase produces NADH, which is used by glyceraldehyde-3-phosphate dehydrogenase in gluconeogenesis. If NADH is not produced in this way, for example, when gluconeogenesis from amino acids occurs, then malate synthesised from pyruvate is exported from the mitochondria and converted to OAA in the cytosol, which generates NADH; OAA is then metabolised by cytosolic PEPCK. The abundance of cytosolic PEPCK, unlike, in general, that of mitochondrial PEPCK, changes greatly in response to factors such as diet and hormones. The proportion of cytosolic to mitochondrial PEPCK in the liver of different species and at different stages of development correlates with how much lactate is produced. For example, birds produce lots of lactate during flight, and contain predominantly mitochondrial PEPCK in the liver (for a review see Hanson and Patel, 1994). Similarly, in fish, Suarez and Mommsen (1987) have proposed that PEPCK is important in gluconeogenesis in the livers of rainbow trout and Atlantic salmon, in which the enzyme is entirely mitochondrial (Mommsen *et al.*, 1985; Mommsen, 1986). Many fascinating adaptations of the Cori cycle (transport of lactate from muscle to the liver, gluconeogenesis in the liver and movement of sugar to the muscle) are found in different vertebrates, and in some of these gluconeogenesis may occur to some extent in the muscle (see Hochachka, 1982). In true herbivores the alimentary tract is modified so that passage of food is delayed and in those specialised structures micro-organisms digest plant components such as cellulose. In ruminants such as cattle, large amounts of propionate and lactate are produced by micro-organisms by anaerobic fermentations in the rumen (a specialised stomach). These are the major

source of energy for these animals, and after absorption into the blood are converted to sugars by gluconeogenesis in the liver (Newsholme and Start, 1973). The other quantitatively important substrates for gluconeogenesis in vertebrates are amino acids (Jungas *et al.*, 1992), and glycerol (which arises from fats) (Newsholme and Start, 1973). These substrates are important, for example, during fasting or after a meal. The reader is referred to Horton *et al*. (2002) for an introduction, and Newsholme and Start (1973) and Hanson and Mehlman (1976) for detailed accounts of vertebrate gluconeogenesis. It should be noted that vertebrates do not generally use fatty acids in gluconeogenesis, unlike plants, fungi and bacteria, because they lack the glyoxylate cycle. An exception to this gluconeogenesis from propionyl CoA (Horton *et al.*, 2002).

2. Micro-organisms

When many bacteria, fungi and protoctists are grown in the presence of gluconeogenic substrates, such as organic or amino acids, and in the absence of sugars, gluconeogenesis occurs. In the 1960s it was found that mutants of a number of species that lacked PEPCK could not grow on these gluconeogenic substrates (Utter and Kolenbrander, 1972 and references therein). However, in some species, under certain conditions, the absence of PEPCK can be overcome by an increase in flux through alternative pathways; for example, in *E. coli* the pathway used is malic enzyme together with PEP synthase (Goldie and Sanwal, 1980). PEP synthase has not been found in flowering plants. However, it catalyses a similar reaction to that of pyruvate, orthophosphate dikinase – an important enzyme of the C4 cycle in flowering plants (Hatch and Slack, 1969; Utter and Kolenbrander, 1972). The function of pyruvate, orthophosphate dikinase remains uncertain in most tissues in flowering plants, even though it is sometimes present at high abundance (for a review see Matsuoka, 1995). In many micro-organisms the presence of PEPCK is dependent on the nature of the culture medium; in the case of some micro-organisms glucose inhibits and gluconeogenic substrates enhance the presence of PEPCK. However, this is not an all or nothing response. In *E. coli* (Sauer *et al*., 1999), *Bacillus subtilis* (Sauer *et al*., 1997) and *Corynebacterium glutamicum* (Petersen *et al*., 2001) PEPCK is sometimes present when cultured in the presence of glucose. The presence of PEPCK depends on a number of factors, such as concentration of glucose and rate of bacterial growth. NMR techniques have shown that in *E. coli* and *B. subtilis* a futile ATP-dissipating cycle occurs between PEPC and PEPCK, the magnitude of which depends on the growth conditions (Sauer *et al*., 1999). A similar cycle occurs in *C. glutamicum* strains that over- or under-expressed PEPCK. In strains with about a seven-fold increase in PEPCK activity, flux through the reaction *in vivo* was only doubled. This effect was due to a decrease in intracellular OAA concentration limiting flux through the PEPCK reaction. In the same strain, although the amount of PEPC activity was not changed, flux through the reaction *in vivo* increased three-fold. This was proposed to be a result of a three-fold decrease in the amount of pyruvate kinase present, which would be likely to increase the concentration of PEP, and decrease in the concentration of aspartate,

an inhibitor of PEPC. Overall, although PEPCK activity was increased several-fold, little change occurred in overall flux between OAA and PEP. In wild type *C. glutamicum* this futile cycle may be important in maintaining the concentrations of the OAA and PEP pools within the cell in the presence of environmental challenges (Petersen *et al.*, 2001). Similar observations with regard to the ability of central metabolism to counteract changes brought about by altering the abundance of enzymes have been made in flowering plants; for example, a reduction in cytosolic pyruvate kinase in tobacco had no detectable effects under normal conditions. Under low light, however, growth was inhibited (see Dennis *et al.*, 1997). Similar results have been obtained with citrate synthase and NAD–malic enzyme (see Hill, 1997). In general, these observations illustrate the resilience of central metabolism in counteracting changes brought about by a change in the abundance of many enzymes, and show that only under certain environmental or developmental conditions is the ability of the cell to counteract these changes overcome. These observations also illustrate that when designing experiments to investigate the effects on metabolism of over- or under-expressing an enzyme in flowering plants, careful consideration of what is known about microbial systems should be taken into account.

3. *Flowering Plants*

In flowering plants gluconeogenesis has been most studied in germinating seeds, in which amino acids and fats are converted to sugars (see Stewart and Beevers, 1967). Gluconeogenesis occurs in the photosynthetic metabolism of leaves of CAM plants, in which organic acids accumulated at night in the vacuole are decarboxylated during the day. The CO_2 is assimilated in photosynthesis and PEP or pyruvate is converted to carbohydrate, which is subsequently used in the synthesis of organic acids during the night (Christopher and Holtum, 1996). The conversion of the lipid and protein components of leaves to sugars for export by gluconeogenesis has been proposed to occur in senescing leaves; however, this seems unlikely (Chen *et al.*, 2000). During the ripening of grape and tomato, evidence has been provided that organic acids are converted to sugars by gluconeogenesis, which utilises PEPCK (see Ruffner and Kliewer, 1975; Bahrami *et al.*, 2001). In addition, it is possible that gluconeogenesis from amino/organic acids is far more widespread than is generally thought in plants and plays a role in the metabolic regulation of pH (see section IX.F).

Germinating seeds

Seeds contain storage reserves which are used to sustain germination and growth of the young plant. During and after the germination of many seeds, stored lipid and carbon skeletons of amino acids are converted to sugars by gluconeogenesis and then transported to the young plant to sustain its growth. PEPCK was found in the cotyledons of germinating lupine seeds by Mazelis and Vennesland (1957), who suggested that it could be involved in the conversion of organic acids to sugars in some tissues. The finding that the glyoxylate cycle occurred in germinating seeds (Kornberg and Beevers, 1957; Beevers, 1993), together with the

observations that in these seeds the sugars are synthesised from lipids (Canvin and Beevers, 1961) and that PEP, rather than pyruvate, is an intermediate in the synthesis of these sugars (Neal and Beevers, 1960), raised the possibility that this gluconeogenic flux was catalysed by PEPCK. Evidence for this was provided by the observations that PEPCK was abundant in the endosperm of germinating castor and that, in this tissue, radiolabelled malate was converted to sugars (Benedict and Beevers, 1961). This became established as the first known function of PEPCK in flowering plants. Although many studies were done on the glyoxylate cycle and gluconeogenesis in germinating seeds in the 1960s and early 1970s, few studies of PEPCK were performed. Cooper and Beevers (1969) showed that the abundance of PEPCK was sufficient to account for the gluconeogenic flux in castor bean. Thomas and ap Rees (1972) characterised the changes in abundance of lipid, sugars and some enzymes during the germination of marrow, and produced an estimate of the rate of gluconeogenic flux. Using subcellular fractionation techniques evidence was provided that the conversion of PEP to sugars occurred in the cytosol (ap Rees *et al.*, 1974). Subsequently, evidence was provided that PEPCK was also located in the cytosol (Leegood and ap Rees, 1978a). In a similar way to gluconeogenesis in micro-organisms and liver (Utter and Kolenbrander, 1972 and references therein), flux through the PEPCK reaction in germinating seeds is altered to a large extent by changes in the abundance of PEPCK. The abundance of PEPCK in germinating marrow correlated with the conversion of lipid to sugars (Leegood and ap Rees, 1978a), increasing about 40-fold during germination and then declining to very low abundance or zero. Later work showed that the increase in the abundance of PEPCK protein matches changes in the abundance of its mRNA (Kim and Smith, 1994). In a similar way to gluconeogenesis in vertebrates (Di Tullio *et al.*, 1974; Jomain-Baum *et al.*, 1976) and photosynthesis in leaves of PEPCK/NAD–malic enzyme type C4 plants (Ray and Black, 1976), the inhibitor of PEPCK activity 3-mercaptopicolinic acid was found to inhibit gluconeogenesis (Leegood and ap Rees, 1978a).

Senescence

In senescing leaves of barley the enzymes of the glyoxylate cycle (isocitrate lyase and malate synthase) appear (Gut and Matile, 1988). The glyoxylate cycle produces organic acids from lipids (for the pathway see Horton *et al.*, 2002). It was suggested that a gluconeogenic flux from lipid occurred (Gut and Matile, 1988). A number of studies confirmed the induction of enzymes of the glyoxylate cycle in senescing or starved tissue (see Chen *et al.*, 2000). Kim and Smith (1994) found that an increase in the abundance of PEPCK mRNA occurred in senescent cucumber cotyledons; however, they expressed reservations as to whether it was involved in gluconeogenesis. Nevertheless, it is widely believed that there is a gluconeogenic flux from lipids to sugars in senescing or starved leaves or cotyledons which utilise PEPCK (Buchanan-Wollaston, 1997; Dangl *et al.*, 2000). This is very unlikely (Chen *et al.*, 2000). The observation that PEPCK mRNA increases in senescing cucumber cotyledons (Kim and Smith,

1994) is probably a result of its persistence in specialised cell types such as trichomes, and a fall in the total mRNA in the cotyledon (Chen *et al.*, 2000). In all senescing or starved leaves or cotyledons studied, PEPCK was not present, except in some cases in specialised cells such as trichomes (Chen *et al.*, 2000). Therefore PEPCK is unlikely to catalyse a gluconeogenic flux in such tissues or cotyledons. The formation of acetyl CoA by fatty acid breakdown, its use by the glyoxylate cycle and the conversion of organic acids to sugars, are sometimes coupled together, as in germinating seeds. If gluconeogenesis is inhibited in germinating seeds, organic acids are metabolised in part by the Krebs cycle (Trevanion *et al.*, 1995b). Similarly, if the glyoxylate cycle is blocked, acetyl CoA arising from lipid breakdown is metabolised by the Krebs cycle (Eastmond *et al.*, 2000).

This raises the question as to the function of the glyoxylate cycle in senescing leaves. The induction of the glyoxylate cycle enzymes is more related to a starvation response than to senescence, because illumination or provision of sucrose massively inhibits their appearance in senescing tissue (McLaughlin and Smith, 1994; Chen *et al.*, 2000). This is not so straightforward, because the tissue often still contains sucrose, so the starvation signal may be related to the metabolism of sugars and not the presence of storage forms (McLaughlin and Smith, 1994). In yeast (*Saccharomyces cerevisiae*) the abundance of enzymes of the glyoxylate cycle is reduced by sugars through a mechanism that involves the phosphorylation of glucose by hexokinases PI and PII (Rose *et al.*, 1991 and references therein). Evidence was obtained that this mechanism might operate in plants (Sheen, 1990). Experiments with protoplasts fed with different analogues of glucose, one of which is phosphorylated by hexokinase and not further metabolised, suggested that the starvation response may be linked to the phosphorylation of glucose by hexokinase. However, this mechanism is poorly understood (Graham *et al.*, 1994). Similarly, in bacteria grown on acetate one function of the glyoxylate cycle is anaplerotic replenishment of the Krebs cycle (see Dawes *et al.*, 1986). In starved protoplasts there will be a shortage of sugars and therefore a shortage of PEP, which is the substrate for PEPC – the enzyme involved in forming OAA for the anaplerotic replenishment of the Krebs cycle. In this situation the glyoxylate cycle can provide succinate for this anaplerotic replenisment (Graham *et al.*, 1994). In starved leaves, proteins are broken down, and many amino acid interconversions occur (Thomas, 1978). In this situation the glyoxylate cycle is likely to serve the same purpose as in starved protoplasts, because intermediates from the Krebs cycle may be used in these interconversions, and an anaplerotic replenishment of the Krebs cycle may be required (Chen *et al.*, 2000). Interestingly, the glyoxylate cycle enzymes are present in many developing seeds and young leaves, in which their functions are unknown (Turley and Trelease, 1990; Nieri *et al.*, 1997). In developing seeds one possibility worth considering is that its presence is a component of a mechanism that reduces a build-up of CO_2. The glyoxylate cycle omits the CO_2-releasing reactions of the Krebs cycle. Removal of CO_2 from developing seeds by diffusion could be a potential problem in some situations. In this context Smith

and ap Rees (1979) showed that even in well aerated soil there was fermentation in the pea root apex. Some aspects of the build-up of CO_2 in seeds and fruit are discussed by Latzko and Kelly (1983) and Blanke and Lenz (1989). It seems possible that just as plants have developed metabolic adaptations to assist in the acquisition of CO_2 for photosynthesis, they may have developed as yet very poorly understood mechanisms to cope with an overabundance of CO_2 and a limited supply of O_2.

CAM

In CAM plants, organic acids are decarboxylated during the day to provide CO_2 for the synthesis of sugars by photosynthesis. Depending on the species, either PEPCK or the malic enzymes are responsible for this decarboxylation (Dittrich *et al*., 1973). At night these organic acids are synthesised by PEPC. The question arises as to the fate of PEP and pyruvate produced by decarboxylation in the day and the source of PEP for the synthesis of organic acids by PEPC at night. The answer is that PEP and pyruvate are largely converted to sugars by gluconeogenesis during the day and sugars converted to PEP at night by glycolysis. The details of this process are dependent on the species (Christopher and Holtum, 1996).

Fruit

Fruit and their developing seed contain many tissues, and querying the function of PEPCK in fruit is rather like enquiring as to its purpose in vertebrates or flowering plants. Detailed immunohistochemistry studies in grape illustrate this point (Walker *et al*., 1999; Famiani *et al*., 2000). In fruit PEPCK is likely to be a component of a number of metabolic processes. These functions are dependent on the type of fruit and its stage of development. Many fruit accumulate large amounts of organic acids during development, which are subsequently dissimilated during ripening. In some fruit gluconeogenesis from stored organic acids is likely to be one function. However, in at least ripening grape, this gluconeogenic flux accounts for a very small amount of the accumulated sugar, and a substantial proportion of the dissimilated organic acids are used by the Krebs cycle rather than gluconeogenesis (Ruffner and Hawker, 1977). The key point to note in ripening fruit is that if malate or citrate enter the Krebs cycle and no Krebs cycle intermediates leave then there will be a build-up in these intermediates. The process of cataplerosis, that is the removal of Krebs cycle intermediates, is necessary. In ripening fruit PEPCK and malic enzyme fulfil this function. For many decades, there have been many studies of the content and type of organic acid in fruit during development (for a review see Ulrich, 1970), but, a great many aspects of the related enzymology remain unknown. PEPC is known to be important in the synthesis of organic acids in many fruit (Ruffner, 1982; Moing *et al*., 2000). In ripening fruit, dissimilation of organic acids may be brought about by the malic enzymes and/or PEPCK. However, their relative contributions in the dissimilation of organic acids in different fruit is uncertain.

PEPCK was first found in fruit, the squash, by Mazelis and Vennesland (1957). The first detailed study of PEPCK in fruit was by Ruffner and Kliewer (1975), who found about 15 μmol h^{-1} g (fresh wt)$^{-1}$. Similarly, Blanke *et al.* (1988) reported that PEPCK was present in apple, kiwi fruit and aubergine. In grape there is a large decline in malate during ripening, which coincides with the accumulation of sugars (Ruffner and Hawker, 1977; Ruffner, 1982; Famiani *et al.*, 2000 and references therein). In the 1960s, ^{14}C feeding of grapes showed that malate was converted to sugars by gluconeogenesis (Ruffner and Kliewer, 1975, and references therein). More rigorous studies, which employed the malate precursor, fumarate-2,3-^{14}C, in order to reduce label in sugars arising from photosynthetic reassimilation of CO_2 released from the C4 or C1 positions of malate, supported the idea of a gluconeogenic flux. Further support for gluconeogenesis was provided by the observation that the incorporation of radiolabel into sugars correlated with the rate of dissimilation of organic acids during development (Ruffner *et al.*, 1975). The fact that the abundance of PEPCK measured by Ruffner and Kliewer (1975) did not correlate with organic acid dissimilation is partly due to the fact that seeds were not removed from the berry, and these contain substantial PEPCK activity (Walker *et al.*, 1999). In grape the rate of dissimilation of organic acids is about 0.3 μmol h^{-1} g (fresh wt)$^{-1}$ and the amount of PEPCK is about 9 μmol h^{-1} g (fresh wt)$^{-1}$. Overall Ruffner and his co-workers provided strong evidence that gluconeogenesis from organic acids occurred in grape. However, it must be stressed that the contribution of gluconeogenesis to sugar accumulation during ripening of grape is at most 5% (Ruffner and Hawker, 1977). In tomato, a similar picture has emerged; radiolabelling studies showed that gluconeogenesis from organic seeds occured (Farineau and Laval-Martin, 1977; Halinska and Frenkel, 1991). During the ripening of tomato there is a decline in both organic acids, cytosolic NADP-malic enzyme and mitochondrial NAD-malic enzyme (Knee and Finger, 1992; Bahrami *et al.*, 2001). In contrast, PEPCK mRNA, protein and activity appeared at the onset of ripening. In tomato the dissimilation of organic acids is about 0.05 μmol h^{-1} g (fresh wt)$^{-1}$ and the amount of PEPCK is about 4 μmol h^{-1} g (fresh wt)$^{-1}$. In comparison, in the cotyledon of germinating marrow the rate of gluconeogenesis is about 3.6 μmol h^{-1} g (fresh wt)$^{-1}$ and the activity of PEPCK is about 420 μmol h^{-1} g (fresh wt$^{-1)}$. However, it should be noted that these cotyledons contain about 50 mg protein g (fresh wt)$^{-1}$ and that tomato flesh contains 1 mg protein g (fresh wt)$^{-1}$ (Knee and Finger, 1992). This is because many fruit possess large cells, a substantial proportion of which is vacuole. It should be noted that in grape and tomato the malic enzymes are also important in the dissimilation of organic acids during ripening. The relative importance of each is uncertain and is likely to depend on a number of environmental and physiological factors. For example, in grape an increase in temperature from 20 to 30°C reduces the conversion of malic acid to sugar, which is associated with an increase in respiration (Ruffner and Kliewer, 1975; Ruffner, 1982).

A detailed study of the abundance of PEPCK in different fruit throughout development was perfomed recently (Walker *et al.*, 1999; Famiani *et al.*, 2000).

This study suggests that the situation in many fruit is more complex than in tomato. Immunoblots of SDS–PAGE gels were used to support measurements of PEPCK activity. In addition, in grape, immunohistochemistry studies were done in conjunction with dissection of different parts of the fruit in which enzyme abundance was measured by activity assay and immunoblots. In grape PEPCK is often abundant in certain cell types, such as the vasculature (Famiani *et al*., 2000). In these it may play a role in the regulation of intracellular pH, which is discussed in section IX.F. More PEPCK was present in the flesh of ripe orange, pineapple, cherries, plums, peach and blueberries (about 0.3 μmol min^{-1} g (fresh $wt)^{-1}$ (R. P. Walker, unpublished observations) than in grape and tomato. PEPCK was not detected in the flesh of ripe apples, pears, banana, raspberries and strawberries. In different fruit PEPCK appears at different times during their development, and in many it appeared before ripening. In plum and cherry, PEPC declines greatly in the flesh before a large proportion of the organic acids have accumulated. Concomitant with the decline of PEPC, PEPCK appeared in the flesh; therefore, the presence of PEPCK and not PEPC correlates with the appearance of organic acids in some situations (R. P. Walker and F. Famiani, unpublished observations). The amount of PEPCK activity that appeared was similar to that of the PEPC that disappeared. In addition, the appearance of PEPCK occurred at the same time as a rapid decline in photosynthetic enzymes, for example RuBisCO. Similarly, in peach flesh the accumulation of organic acids does not always correlate with PEPC protein or activity; however, changes in the abundance of PEPCK were not investigated (Moing *et al*., 2000). PEPCK is also present in peach and shows a similar developmental pattern to cherry and plum (R. P. Walker and F. Famiani, unpublished observations). Fruit are bulky structures, and the environment inside them might present some unusual challenges for metabolism. For example, the supply of oxygen and removal of CO_2 by diffusion might under some circumstances present a problem. If the tissues are photosynthetic then this would help to alleviate this problem. It is widely believed that in some fruit a portion of CO_2 released by the Krebs cycle is refixed by PEPC (Latzko and Kelly, 1983; Blanke and Lenz, 1989). It might be no coincidence that in plum and cherry, PEPCK appearance is correlated with the substantial decline in RuBisCO. A possibility is that PEPCK is important in the synthesis of organic acids in some fruit. This is not a new idea, and has been suggested by Mazelis and Vennesland (1957), and, in the case of grape, by Ruffner and Kliewer (1975). It is worthwhile exploring this possibility a little further.

It is generally believed that PEPC is responsible for the anaplerotic replenishment of the Krebs cycle in flowering plant tissues. While this may be true for a large number of tissues and situations, for others it is unlikely. In at least some starved tissue this role is likely to be fulfilled by the glyoxylate cycle (Graham *et al*., 1994; Chen *et al*., 2000). In some tissues and situations it is possible that PEPCK acts as a carboxylase, and a proportion of the OAA so produced is used by the Krebs cycle. The question arises as to why PEPCK should be used in this way. A comparison of the reactions catalysed by PEPC and PEPCK shows that, unlike

PEPC, the reaction catalysed by PEPCK is reversible and produces ATP. Comparative biochemistry reveals that in a wide range of different organisms PEPCK often acts as a carboxylase in anaerobic fermentations. This may be because, unlike PEPC, the PEPCK reaction produces ATP. Mazelis and Vennesland (1957) suggested 'that in many, if not all, plant tissues, malate should be regarded as the natural end-product of the anaerobic phase of carbohydrate breakdown, analogous to lactate in muscle and ethanol in yeast'. While in many tissues, such as roots deprived of oxygen, this is not true, in some fruit it is. In roots deprived of oxygen, for example, it is important to avoid the problem of intracellular acidification and, towards this end, malate is consumed rather than produced (Sakano, 2001), and PEPCK is not induced in roots under these conditions (Smith, 1985). However, fruit are very different to roots – acidification is not a problem because fruit can accumulate malate, and the protons produced during its synthesis, in the vacuole. Fruit may synthesise malate, a process that does not require oxygen and consumes CO_2, and, by utilising PEPCK in this process, can produce ATP. Another important feature of the PEPCK reaction is that it is reversible, and in some situations this may be very useful as a method of regulating the direction of net flux through the reaction. A critical factor *in vivo* in determining the direction of net flux through the PEPCK reaction is likely to be the concentration of OAA. For example, if there is a lack of O_2 then this might lead to a higher NADH/NAD ratio (Dennis *et al*., 1997); this may then lower the cytosolic concentration of OAA (because of the equilibrium position of the malate dehydrogenase reaction [malate] × [NAD] = constant × [OAA] × [NADH]). If the concentration of OAA falls, then this will enhance the carboxylase activity of PEPCK, so that more ATP is produced and CO_2 is consumed. If O_2 was not limiting, then the net flux through the PEPCK reaction could be biased towards PEP production and gluconeogenesis. The use of PEPCK in this way would enable metabolism to respond rapidly to the concentration of O_2 and CO_2 within the fruit. Therefore, in fruit, there may be a balance between the supply of O_2 and removal of CO_2 and the requirement to produce ATP and NADP(H). This may be a key point in understanding organic acid metabolism in fruit. It is well known that many cultivars of a number of commercially grown fruit possess very different contents of organic acids. It would be interesting and easy to screen ripe fruit of these cultivars to see if any lacked PEPCK. The availability of such a mutant would be very useful in studying the function of PEPCK in fruit metabolism. An interesting point worthy of further consideration is that a number of types of plant cells possess the ability to induce large amounts of PEPCK in response to factors such as low light intensity and/or O_2 supply (Z.-H. Chen, R. P. Walker, unpublished observations).

B. GLYCERONEOGENESIS

The specific activity of PEPCK is higher in white adipose tissue than in liver. A number of lines of evidence support the proposal of Ballard *et al*. (1967) that

PEPCK functions in the generation of 3-glycerophosphate from lactate or pyruvate in this tissue. Fatty acids and 3-glycerophosphate are used to form triglycerides (Hanson and Patel, 1994, and references therein). Glyceroneogenesis is important in adipose tissue and liver during fasting when the availability of glucose is low.

C. PHOTOSYNTHETIC CO_2-CONCENTRATING MECHANISMS

In considering this aspect of the metabolic role of PEPCK it is important to consider the different habitats in which photosynthetic organisms live. Some interesting aspects of the marine environment are described in Levinton (2001). The acquisition of CO_2 is essential for photosynthetic organisms. For land plants the availability of water may present problems with regard to the acquisition of CO_2. Land plants usually obtain CO_2 from the atmosphere, which enters the plant by diffusion through pores such as stomata. However, loss of water vapour by diffusion through stomata is a problem when acquisition of water is limiting. Many plants live in habitats such as deserts, or saline soils, or as epiphytes on trees, in which water supply may present problems. There are number of adaptations that decrease water loss associated with the acquisition of CO_2, for example, C4 photosynthesis and CAM. These have evolved on many occasions in different families of flowering plants (Ehleringer and Monson, 1993). About 10% of land plant species possess these adaptations, of the 250 000 species of land plant there are about 2500 species which are C4 and 25 000 species which are CAM (see Johnston and Raven, 1986). It should be noted that about 20 species of flowering plant, known as C3–C4 intermediates, possess a different CO_2-concentrating mechanism; however, PEPCK is not known to function in these. RuBisCO is the enzyme ultimately responsible for incorporation of CO_2 into sugars by the Benson–Calvin cycle in plants and algae. However, this enzyme can use O_2 instead of CO_2 as a substrate (the oxygenase activity may be a consequence of the enzyme having evolved at time when the earths atmosphere did not contain O_2 (Walker, 1992)). A metabolic pathway, photorespiration, deals with the products of the oxygenase activity. High light intensity and temperature – often characteristics of the habitats of C4 and CAM plants – increase the oxygenase activity of RuBisCO relative to its carboxylase activity. C4 and CAM reduce photorespiration by increasing the concentration of CO_2 at the site of its fixation by RuBisCO (for a detailed account, see Walker 1992; Leegood, 1993; Canvin and Salon, 1997). PEPCK is often a component of the C4 and CAM mechanisms. Another situation in which photorespiration and the acquisition of CO_2 may be a problem is the aquatic environment, both because of the slow diffusion of O_2 and CO_2 in water and their concentration in some habitats (Bowes, 1985; Keeley, 1996). In contrast to land plants, the majority of aquatic species possess some form of mechanism to concentrate inorganic

carbon (Raven, 1984). These mechanisms may involve the uptake of bicarbonate by a membrane carrier, and/or involve the synthesis and dissimilation of organic/amino acids. For example, in the aquatic flowering plant *Hydrilla verticillata*, the synthesis of organic acids in the cytosol utilising PEPC; their subsequent transport into the chloroplast; and decarboxylation by NADP–malic enzyme, act as a CO_2-concentrating mechanism (Magnin *et al*., 1997). In the aquatic environment a number of algae utilise PEPCK as a component of the mechanisms which alleviate these problems.

1. C4 Plants

In most C4 plants, of which maize is a well-known example, CO_2 is fixed into dicarboxylic acids in the mesophyll cells of the leaf. Malate and/or amino acids, produced by transamination, diffuse into a layer of cells underlying the mesophyll called the bundle sheath. In these, decarboxylation occurs and CO_2 enters the Benson-Calvin cycle (for reviews see Hatch, 1987; Edwards *et al*., 2001). PEPC is responsible for the fixation of CO_2 in the mesophyll and, depending on the species, PEPCK and/or chloroplastic NADP–malic enzyme and/or mitochondrial NAD–malic enzyme is responsible for decarboxylation in the bundle sheath. C4 plants were classified into three types: the PEPCK, NAD–malic enzyme and NADP–malic enzyme types, on the basis of the enzyme used to decarboxylate C4 acids in the bundle sheath (Gutierrez *et al*., 1974; Hatch *et al*., 1975; for a historical account see Hatch, 1999). However, this is an oversimplification, and there is at least one further type, and possibly two. One is the PEPCK/NADP-malic enzyme type, an example being maize (Walker *et al*., 1997; Furumoto *et al*., 1999; Wingler *et al*., 1999), the other potentially is an NADP–malic enzyme/NAD–malic enzyme type. It should be noted that Gutierrez *et al*. (1974) first provided evidence for the existence of these further two classes. However, subsequent work led to the widely held view that there are three main types of C4 plants.

PEPCK/NAD–malic enzyme type

PEPCK was first found in leaves of C4 plants by Edwards *et al*. (1971), who proposed that it acted as a decarboxylase in the bundle sheath. Leaves of these C4 plants typically contain about seven units of PEPCK per gram fresh weight (one unit = 1 μmol min^{-1} of substrate converted to product at 25°C, and under other conditions in *in vitro* assay in which maximum activity is measured). NAD–malic enzyme was also found to be present in this group of C4 plants (Gutierrez *et al*., 1974). The C4 pathway in this group was established by a number of studies over many years (Hatch *et al*., 1975; Hatch and Kagawa, 1976; Ray and Black, 1976; Hatch and Mau, 1977; Rathnam and Edwards, 1977; Smith *et al*., 1982; Chapman and Hatch, 1983; Smith and Woolhouse, 1983). A key finding was that both PEPCK and the mitochondrial NAD–malic enzyme contribute to C4 acid decarboxylation in the bundle sheath, and that

ATP for the PEPCK reaction is provided by oxidative phosphorylation, using NADH provided by the action of NAD–malic enzyme (Burnell and Hatch, 1988a,b). Subsequent studies further characterised the role of mitochondrial metabolism in providing ATP for the PEPCK reaction (Carnal *et al*., 1993; Agostino *et al*., 1996). For details of the C4 pathway in these plants the reader is referred to Burnell and Hatch (1988b).

PEPCK/NADP–malic enzyme type

Large amounts of PEPCK are also present in some members of the group thought only to use NADP–malic enzyme as a decarboxylase. One such plant is maize, whose leaves contain about 3.5 units of PEPCK per gram fresh weight (one unit = 1 μmol min^{-1} of substrate converted to product at 25°C, and under other conditions in *in vitro* assay in which maximum activity is measured) (Walker *et al*., 1997). PEPCK was first reported to be present in maize seedlings by Mazelis and Vennesland (1957). However, Slack and Hatch (1967) could not detect PEPCK in maize. Gutierrez *et al*. (1974) reported that some members of the NADP–malic enzyme group contained PEPCK activity, which varied from about 20% of that of NADP–malic enzyme to about 3% in maize, and it was suggested that PEPCK could also contribute to decarboxylation in some members of this group. The problem with measuring PEPCK in extracts of maize leaves is that in *in vitro* assay large amounts of ADP-independent carboxylation of PEP occur. This makes it very difficult to detect PEPCK activity, and it is almost impossible to show reliably that it is present in crude extracts, unless the extraction and assay procedures are modified. However, the availability of a specific antiserum solved this problem, and PEPCK was found to be abundant in maize leaves (Walker *et al*., 1997). Once its presence was known, subsequent refinement of extraction and assay showed that maize leaves contain large amounts of PEPCK activity – about 30% of the activity of NADP–malic enzyme – and in these it was proposed to act as a decarboxylase in the bundle sheath (Walker *et al*., 1997; Furumoto *et al*., 1999). In maize, a detailed study had shown that about 25% of CO_2 fixed in the mesophyll was transferred to the bundle sheath as aspartate, which was then transaminated by mitochondrial aspartate aminotransferase (Chapman and Hatch, 1981 and references therein). However, because it was not known that PEPCK was present, the OAA so produced was proposed to be converted to malate by malate dehydrogenase and decarboxylated by chloroplastic NADP–malic enzyme. Wingler *et al*. (1999) showed that the OAA produced from aspartate is decarboxylated by PEPCK. However, our understanding of the C4 cycle in maize is still far from complete. For example, if aspartate is metabolised by aspartate aminotransferase in the mitochondria, as proposed, then what prevents the OAA so produced from being converted to malate by malate dehydrogenase, which is reported to be present in these mitochondria (Chapman and Hatch, 1981)? In PEPCK/NAD-type C4 grasses, ATP for the PEPCK reaction is provided by the action of mitochondrial NAD–malic enzyme and subsequent oxidative phosphorylation, but this does not appear to occur in maize because this enzyme seems to be at low abundance.

Although this requires further detailed study for confirmation, it raises the question as to the source of ATP for the PEPCK reaction.

Is there a NADP/NAD–malic enzyme type C4 plant?
Gutierrez *et al.* (1974) and Rathnam and Edwards (1975) reported that a number of NADP–malic enzyme type plants also contained NAD–malic enzyme. Subsequent studies led to the widely held belief that the activity attributed to NAD–malic enzyme was in fact that of NADP–malic enzyme (Hatch *et al.*, 1982). However, it may be extremely difficult to distinguish between the different forms of malic enzyme on the basis of the properties of their activity. When leaf extracts are subjected to SDS–PAGE and blots probed with an antiserum to NAD–malic enzyme (Murata *et al.*, 1989) similar amounts of signal are seen in extracts of 'NADP–malic enzyme' dicots, but not in 'NADP-malic enzyme' monocots, as is seen in PEPCK/NAD–malic enzyme types (Fig. 3). This suggests that C4 dicots that use NADP–malic enzyme also contain large amounts of NAD–malic enzyme. Some practical points should be noted. In our hands the antiserum raised against NAD–malic enzyme from *Amaranthus hypochondriacus* (Long *et al.*, 1994) recognises both NAD– and NADP–malic enzyme, these proteins show about 40% sequence identity (Long *et al.*, 1994). It is unlikely that the signal shown in Fig. 3 is a cross-reaction with NADP-malic enzyme, because this antiserum does not recognise NADP–malic enzyme in C4 monocots and recognises NAD–malic enzyme in leaves of both C3 plants and C4 NAD–malic enzyme type monocots and dicots. A potentially important point is that at least some C4 dicots that use NADP–malic enzyme contain substantially higher activities of asparatate and alanine aminotransferase in the bundle sheath than monocots that use NADP–malic enzyme (Meister *et al.*, 1996). In addition, in *Flaveria bidentis*, a C4 dicot that uses NADP–malic enzyme, large amounts of aspartate are transported to the bundle sheath (Meister *et al.*, 1996). This is potentially significant because NAD–malic enzyme-type plants transport mainly aspartate to the bundle sheath (Hatch *et al.*, 1975). We present these data because of their potential significance and feel that this possibility is worthy of further consideration. If this observation is substantiated then there would be five types of C4 photosynthetic mechanism: those which use only NADP–malic enzyme, those which use only NAD–malic enzyme, those which use a combination of both malic enzymes, those which use PEPCK and NADP–malic enzyme, and those which use PEPCK and NAD–malic enzyme. No C4 plant has so far been shown to use all three decarboxylases, and none to use only PEPCK. The latter is an interesting point because CAM plants that use PEPCK as a decarboxylase are often reported to possess as much malic enzyme as those which use malic enzyme as the sole decarboxylase (Christopher and Holtum, 1996).

2. CAM Plants
A number of plants accumulate organic acids during the night and dissimilate them during the day. In the late 1940s and 1950s an important part of the

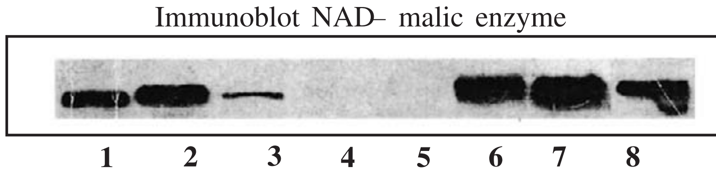


Fig. 3. Immunoblot analysis of NAD–malic enzyme in C4 plants. Extracts of leaves were subjected to SDS–PAGE and immunoblotting. Electrophoretic techniques were performed as described by Walker and Leegood (1996). PEPCK/NAD–malic enzyme type: 1 = *Chloris gayana*; 2 = *Panicum maximum*. PEPCK/NADP–malic enzyme type: 3 = maize (*Zea mays*). NADP–malic enzyme monocots: 4 = *Sorghum vulgare*; 5 = sugar cane (*Saccharum officinarum*). NAD–malic enzyme type monocot: 6 = *Panicum miliaceum*. 'NADP–malic enzyme type' dicots: 7 = *Flaveria bidentis*; 8 = *Gomphrena globosa*.

mechanism responsible was elucidated (see Walker, 1992). In the leaves of many CAM plants, the stomata open at night, CO_2 then enters the leaf and is fixed into organic acids by PEPC. These organic acids are accumulated in the vacuole. During the day, the stomata close and organic acids leave the vacuole, these acids are decarboxylated and the CO_2 released then enters the Benson–Calvin cycle. This mechanism reduces water loss because more water vapour passes through the stomata during the day when temperatures are higher (Ting, 1985). In 1973 Dittrich *et al*. found that PEPCK is present at high abundance in the leaves of many CAM plants, and showed that CAM plants can be divided into two types based on whether PEPCK or the malic enzymes are used to decarboxylate organic acids. Many plants grown in the home, such as *Aloe*, *Hoya* and bromeliads are PEPCK-type CAM plants. In leaves of some CAM plants, such as *Clusia rosea,* the activity of PEPCK is about 4 units per gram fresh weight (Borland *et al*., 1998) (one unit = 1 μmol min^{-1} of substrate converted to product at 25°C and under other conditions in *in vitro* assay in which maximum activity is measured). Subsequent studies confirmed that PEPCK is widely used as a decarboxylase in CAM (Black *et al*., 1996; Christopher and Holtum, 1996, and references therein). In some species different parts of the CAM pathway occur in different tissues (Nishio and Ting, 1987), and in others PEPCK and the CAM pathway are induced by environmental conditions such as drought (Lüttge, 1996; Borland *et al*., 1998).

3. *Aquatic Photosynthetic Organisms*

PEPCK is present in a number of algae. In some it is a component of a mechanism involved in the acquisition of CO_2/reduction of photorespiration; in others it has additional or different functions. The algal groups are a diverse phylogenetic group and are thought to have diverged early in evolution, during the evolution of the protoctista (Margulis and Schwartz, 1998). Flowering and other plants such as mosses, ferns and conifers are thought to have evolved from the

green algae. For an introductory account of the biology of these groups the reader is referred to Raven *et al*. (1992).

Chlorophyta (green algae)

The green algae are a diverse group which contains at least 7000 species, and, although mainly aquatic, some live in other habitats such as on tree trunks and in the soil. Substantial amounts of PEPCK–ATP have been found in *Udotea flabellum,* a green seaweed (Reiskind *et al*., 1988). A detailed study of photosynthetic carbon metabolism strongly suggested that PEPCK is a component of a CO_2-concentrating mechanism. This algae contained large amounts of PEPCK, which was five times higher than the rate of photosynthesis. A reworking of the data would suggest 1–3 units of PEPCK per gram fresh weight (one unit = 1 μmol min^{-1} of substrate converted to product at 25°C, and under other conditions in *in vitro* assay in which maximum activity is measured). However, the activity of other carboxylases and decarboxylases was low (Reiskind *et al*., 1988). Large amounts of malate and aspartate were labelled in illuminated tissue, which was inhibited by the inhibitor of PEPCK 3-mercaptopicolinic acid. This suggested that PEPCK was acting as a carboxylase. On the other hand CO_2 for photosynthesis could be provided by malate as the only source of carbon, and this ability was inhibited by 3-mercaptopicolinic acid. This suggested that PEPCK could also act as a decarboxylase *in vivo*. This raised the possibility that PEPCK acts as a carboxylase in the cytosol, and a decarboxylase in the chloroplast (Reiskind and Bowes, 1991). However, a subsequent immunohistochemistry study suggested that PEPCK was only present in the cytosol (Reiskind and Bowes, 1995). A possible explanation for these observations is as follows. PEPCK is located in the cytosol, in which it can act as a carboxylase or decarboxylase. A futile cycle exists between the carboxylase and decarboxylase activities. The direction of net flux out of the cycle is largely dependent on the concentration of cytosolic OAA and the ATP to ADP ratio. Factors that increase OAA concentration in the cytosol, such as cytosolic malate concentration and an increase in NAD/NADH ratio, would enhance the decarboxylase activity. The physiological relevance of this mechanism would be to assist in the acquisition of inorganic carbon from sea-water and to provide a constant high supply of CO_2 for photosynthesis. A similar mechanism might also be present in brown algae.

Rhodophyta (red algae)

There are about 4000 species of red algae, most are multicellular and marine, living attached to rocks. PEPCK–ATP has been reported in some red seaweeds; however, its function is unknown (Holbrook *et al*., 1988; Israel *et al*., 1991).

Dinomastigota (dinoflagellates)

Most dinoflagellates are unicellular organisms; there are about 2100 species which are members of plankton in the sea and fresh water. Some species are heterotrophic. Dinoflagellates have been found to contain pyruvate carboxylase

(Appleby *et al.*, 1980) and PEPCK–ATP (Descolas-Gros and Oriol, 1992). A heterotrophic species was reported to fix inorganic carbon by PEPCK in the dark (Descolas-Gros and Oriol, 1992).

Euglenophyta

There are about 1000 species of euglenoids and these occur in fresh water, and all are unicellular, apart from one colonial genus. About one-third are autotrophic and the rest heterotrophic. They may have arisen from the incorporation of green algae into a zoomastigote protozoan (Raven *et al.*, 1992). Perhaps for this reason *Euglena gracilis* contains PEPCK–GTP and not PEPCK–ATP (Pönsgen-Schmidt *et al.*, 1988). The abundance of PEPCK in *E. gracilis* is greatly altered by culture conditions, and is substantially increased in heterotrophic growth in the dark as compared with autotrophic conditions (Laval-Martin *et al.*, 1981; Pönsgen-Schmidt *et al.*, 1988). PEPC is present in *E. gracilis* and shows opposite changes in abundance on transition from autotrophic to heterotrophic growth conditions (Laval-Martin *et al.*, 1981; Pönsgen-Schmidt *et al.*, 1988). The function of PEPCK during heterotrophic growth is dependent on culture conditions: if *E. gracilis* is grown on lactate, PEPCK acts as a decarboxylase and catalyses a gluconeogenic flux from the lactate (Briand *et al.*, 1981). On the other hand, if it is cultured in the presence of glucose anaerobically, it is proposed to act as a carboxylase and to be important in fermentation reactions leading to the production of wax monoesters (Pönsgen-Schmidt *et al.*, 1988). PEPCK–GTP was purified from *E. gracilis* to a specific activity of 20 units per mg of protein (one unit = 1 μmol per min), and was shown to be pure by SDS–PAGE (Pönsgen-Schmidt *et al.*, 1988). However, the molecular weight was about three times higher than from other organisms: this surprising observation requires further investigation.

Bacillariophyta (Diatoms)

The diatoms are mostly unicellular and are very abundant members of plankton in both the sea and freshwaters. There are at least 5600 living species. Most diatoms are autotrophic; however, many can become heterotrophic under certain conditions (Raven *et al.*, 1992). PEPCK–ATP was first found in diatoms by Holdsworth and Bruck (1977). PEPCK–ATP was purified from a diatom and reported to be homogeneous and to have a molecular weight of 62 kDa. However, the specific activity was 1000 times less than that of PEPCK–ATP from other organisms (Holdsworth and Bruck, 1977). The reason for this surprising result needs further investigation. The presence of PEPCK–ATP in diatoms was confirmed by subsequent studies (Kremer and Berks, 1978; Appleby *et al.*, 1980; Descolas-Gros and Oriol, 1992). These studies and Beardall (1989) provided evidence that PEPC and pyruvate carboxylase are absent in diatoms. Diatoms fed $^{14}CO_2$ rapidly incorporate substantial label into malate and/or amino acids in addition to 3-phosphoglycerate formed by the Benson–Calvin cycle (Beardall, 1989; Johnston *et al.*, 2001, and references

therein). However, label in C4 compounds did not appear before label in 3-phosphoglycerate (see, for example, Fig. 4; Mortain-Bertrand *et al.*, 1987) which suggested that a C4 pathway similar to that in flowering plants was not present. These observations led to the widely held view that PEPCK acts as a carboxylase. However, Reinfelder *et al.* (2000) reported PEPC to be present. It is important to establish which enzymes capable of carboxylating PEP or pyruvate and decarboxylating OAA or malate are present in diatoms, and under what culture conditions. The ratio of labelling of malate and/or amino acids to 3-phosphoglycerate increases under low light (Beardall, 1989 and references therein). In the sea, diatoms are subjected to rapid changes in light intensity as a result of circulation of water from the surface to lower depths (Mortain-Bertrand *et al.*, 1987; Levinton, 2001). At lower depths where light intensity is low, the synthesis of ATP by the carboxylase activity of PEPCK may be important (Holdsworth and Bruck, 1977). This may be a similar situation to organisms adapted to cope with anaerobiosis, which use PEPCK to generate ATP by substrate-level phosphorylation, because when the respiration of diatoms exceeds photosynthesis the O_2 supply may be limiting in some habitats. It has been proposed that in diatoms the main role of PEPCK is anaplerotic (see Johnston *et al.*, 2001). However, this could depend on the species and culture conditions, as in the Euglenophyta. This requires further investigation. In the light, PEP for the PEPCK reaction is provided by the Benson–Calvin cycle, and, in the dark, by breakdown of reserve carbohydrate (Mortain-Bertrand *et al.*, 1987). The amount of PEPCK and the amount of light-independent fixation of CO_2 in diatoms are greatly influenced by the species and culture conditions (Mortain-Bertrand *et al.*, 1987; Descolas-Gros and Oriol, 1992). For example, the content of PEPCK in a diatom cultured under

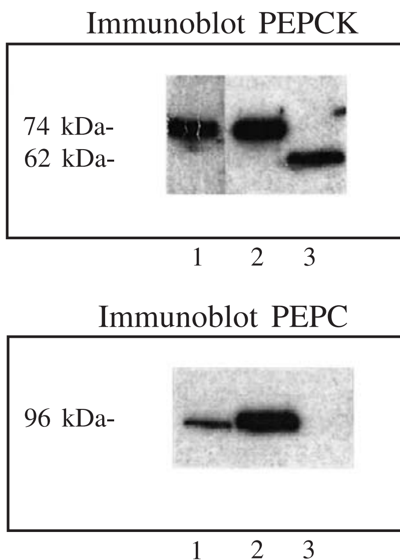


Fig. 4. Immunoblot analysis of PEPCK and PEPC in brown algae. Extracts of tissues were subjected to SDS–PAGE and immunoblotting. Antibodies used are described in Walker and Leegood (1996) and Walker *et al.* (2002). Electrophoretic techniques were performed as described by Walker and Leegood (1996). 1 = cucumber cotyledons; 2 = maize leaf; 3 = *Fucus serratus*.

continuous light was about 20 times less than when grown under a two-hour light–two-hour dark photoperiod (Mortain-Bertrand *et al*., 1987). A recent study which suggested that PEPCK is a component of a C4-like photosynthetic CO_2-concentrating mechanism under certain conditions (Reinfelder *et al*., 2000), similar to that proposed for the aquatic flowering plant *Hydrilla verticillata* (Magnin *et al*., 1997) and the green algae *Udotea flabellum* (Reiskind and Bowes, 1991), is strongly disputed (Johnston *et al*., 2001). Reinfelder *et al*. (2000) proposed that PEPC acts as a carboxylase in the cytosol to synthesise malate, which was subsequently decarboxylated in the chloroplast by PEPCK. This proposal needs more experimentation to substantiate it. It is possible that under certain conditions PEPCK may function in the same way as we suggest for brown algae in the next section.

Phaeophyta (brown algae)

There are about 1500 species of brown algae, many of which are brown seaweeds. These often possess a high degree of tissue specialisation (see Chapman and Chapman, 1973). Brown algae are almost exclusively found in the sea, and range in size from minute discs to kelps of many tens of metres in length. PEPCK–ATP was first found in brown algae by Akagawa *et al*. (1972). Since this report PEPCK has been subject to much study in this group. Brown algae are capable of high rates of light-independent fixation of CO_2, which varies between 1 and 8% of the photosynthetic rate when whole organisms are considered, and up to 85% in the meristem of *Laminaria hyperborea* (Kremer, 1981). This figure depends on a number of factors – such as the developmental status of the thallus, the part of the thallus which is sampled, the temperature, light intensity and supply of inorganic carbon. Brown algae contain appreciable amounts of PEPCK, for example, *Ascophyllum nodosum* contains 0.8 units per gram fresh weight (Johnston and Raven, 1989); similar values were reported for three species of *Fucus* (Küppers and Kremer, 1978; one unit = 1 μmol min^{-1} of substrate converted to product at 25°C, and under other conditions in *in vitro* assay in which maximum activity is measured). Furthermore, because of the low protein content of brown algae, its abundance on a protein basis is similar to that of the enzyme from leaves of C4 flowering plants (cf. Johnston and Raven, 1989; Walker *et al*., 2002). In contrast, activities of other potential carboxylases: PEPC, PEP carboxytransphosphorylase and pyruvate carboxylase are reported to be low or absent (Kremer and Küppers, 1977; Kerby and Evans, 1983). These observations led to the widely held view that PEPCK was responsible for the light-independent fixation of CO_2 in brown algae. It should be noted that PEPCK also fixes CO_2 in the light (Kremer, 1981, 1984). In *A. nodosum*, light-independent fixation of CO_2 in the light was much greater than in the dark (see Johnston and Raven, 1989). Experiments using ^{14}C suggested that under darkness PEP is provided by breakdown of mannitol and subsequently glycolysis, whereas in the light it is provided by 3-phosphoglycerate – the primary product of photosynthesis (Kremer, 1981, 1984).

In *Laminaria digitata*, *L. hyperborea*, *L. saccharina* and *Fucus vesiculosus*, the ratio of the amount of PEPCK to RuBisCO varies greatly between different parts of the thallus. The ratio of incorporation of $^{14}CO_2$ by light-independent fixation to that of photosynthesis was similar to the ratio of PEPCK to RuBisCO in different parts of the thallus (Küppers and Kremer, 1978). In general the highest PEPCK activity was associated with growing regions, and RuBisCO with the mature thallus. In early spring, the blade of *L. hyperborea* starts to grow; however, light intensity is less than that required to sustain its growth (Lünning and Dring, 1979; Küppers and Weidner, 1980). In this situation, PEPCK is proposed to have an anaplerotic role. To sustain growth, stored mannitol is metabolised by glycolysis, and a proportion of the PEP so produced is carboxylated by PEPCK. The OAA is used to replenish the Krebs cycle (Weidner and Küppers, 1982). PEPCK was proposed to be used as a carboxylase because, unlike PEPC, it produces ATP (Weidner and Küppers, 1982).

However, in brown algae PEPCK is likely to have additional roles. The observation that O_2 has little inhibitory effect on the photosynthesis of brown algae (Kremer, 1980 and references therein) raised the possibility that PEPCK may play a role in a photosynthetic CO_2-concentrating mechanism. High short-term fixation of ^{14}C into malate and aspartate led to the suggestion that a C4 cycle operated in brown algae (Weidner and Küppers, 1973; Joshi *et al*., 1974). However, subsequent work showed that the label from malate and aspartate was not rapidly transferred to sugars, which showed that brown algae were not C4 plants (Kremer and Küppers, 1977). The possibility that the photosynthetic CO_2-concentrating mechanism was similar to CAM was raised by the observation that there was an increase in titratable acidity during the night in *A. nodosum* that is paralleled by an increase in malate (Kerby and Raven, 1985; Johnston and Raven 1987). These observations were consistent with a mechanism that is in some ways similar to CAM (Kerby and Raven, 1985; Johnston and Raven, 1987). This view is supported by the observations of Axelsson *et al*. (1989a,b) and Axelsson (1991), who showed that when the uptake of carbon from the medium was inhibited, CO_2 for photosynthesis was likely to be provided by its release from an internal store. In brown algae, light-saturated photosynthesis in red light can be increased up to five-fold by blue light, and it has been demonstrated that under red light photosynthesis is not saturated by the concentration of inorganic carbon in normal sea-water (Schmid and Dring, 1996a,b and references therein). However, the rate of photosynthesis is increased when the concentration of inorganic carbon in sea-water is increased, and this abolishes the stimulation of photosynthesis by blue light. These results led to the suggestion that blue light leads to the mobilisation of a storage metabolite formed by the light-independent fixation of CO_2, and that this mobilisation releases CO_2, which is used in photosynthesis (Schmid and Dring, 1996a,b).

As pointed out by Schmid and Dring (1996a,b) much more work is needed to establish the enzymatic basis of this mechanism. In brown algae, PEPC is not thought to be present, low activities of a malic enzyme have been reported

(Akagawa *et al.*, 1972), and pyruvate orthophosphate dikinase is absent. However, in many tissues it is very difficult to reliably measure the activity of some enzymes; for example, it was difficult to establish the presence of PEPCK in maize leaves and the proposed presence of NAD–malic enzyme in the leaves of NADP–malic enzyme monocots. In addition, it is very difficult to prepare extracts of brown algal tissues for subsequent analysis (Marsden *et al.*, 1981; Kerby and Raven, 1985). When blots of SDS–PAGE gels loaded with brown algal extracts were probed with antibodies raised against PEPCK from flowering plants (Walker et al., 2002) the antibody strongly recognised the brown algal enzyme (Fig. 4). PEPCK from *Fucus serratus* was found to have a molecular mass of 62 kDa after SDS–PAGE and immunoblotting (Fig. 4). Similar results were obtained with *F. vesiculosus* and *L. saccharina* (R. P. Walker, unpublished observations). For *F. serratus*, the 62 kDa band copurified with PEPCK activity during both ammonium sulphate fractionation and ion-exchange chromatography (R. P. Walker unpublished observations). PEPC was not detected on a blot of an extract of *F. serratus* probed with an antibody against the enzyme from a flowering plant (Fig. 4). This was probably because the enzyme was not present because no PEPC activity could be detected in an extract of the brown algae, but it could be found if it was coextracted with the leaf of cucumber, which suggests that PEPC was not inactivated upon extraction of the brown algal tissues (R. P. Walker, unpublished observations). Similar results were obtained with *F. vesiculosus* and *L. saccharina* (R. P. Walker, unpublished observations). This shows that at least some of the many available antibodies raised to enzymes from flowering plants are suitable for use with these algae. The immunoblot suggests that PEPCK from brown algae does not have the N-terminal extension possessed by the enzyme from flowering plants. A similar size was reported for the enzyme from the brown algae *A. nodosum* by gel filtration and SDS–PAGE of the purified protein, which suggests that the enzyme is a monomer (Kerby and Evans, 1983). However, as pointed out by Johnston and Raven (1989) the value of the specific activity of this purified enzyme was very low – about 200 times less than that of PEPCK–ATP from other sources. Whether this is a result of inactivation after extraction is unknown. Johnston and Raven (1989) raised the point that the affinity of PEPCK for CO_2 was very low compared with the *in vivo* concentration of CO_2, and that it would be insufficient to account for non-photosynthetic fixation of CO_2 in the light by PEPCK in *A. nodosum*. This is no longer a problem because the low affinity of PEPCK in *in vitro* assay is a result of unphysiological assay conditions (Chen *et al.*, 2002). An important point is that the carboxylase activity of PEPCK–ATP from both the kinetoplastid *Trypanosoma cruzi* (Urbina, 1987) and the flowering plant *Panicum maximum* (Walker *et al.*, 2002) is greatly inhibited by ATP. Interestingly, at an ATP to ADP ratio of 4, the inhibition of both enzymes was about 90%, and at a ratio of 1 it was 80% (fig. 6 in Urbina, 1987; fig. 3 in Walker *et al.*, 2002). At least for the flowering-plant enzyme, these values were similar at different PEP concentrations (fig. 3 in Walker *et al.*, 2002). Similarly, PEPCK from the brown algae *L. hyperborea* is inhibited about 80% at

a ATP to ADP ratio of 1 (fig. 5 in Weidner and Küppers, 1982). Johnston and Raven (1989) found that the rate of non-photosynthetic fixation of CO_2 in *A. nodosum* was 3.0 μmol h^{-1} g (fresh wt)$^{-1}$ in the light, and that maximum activity of PEPCK was about 48 μmol h^{-1} g (fresh wt)$^{-1}$. Even if PEPCK was inhibited 90% by a ratio of ATP to ADP of 4, this would leave 4.8 μmol h^{-1} g (fresh wt)$^{-1}$ PEPCK activity. In the cytosol of mammalian and flowering plant cells, a ratio of ATP to ADP of 3 to 8 is commonly found (Newsholme and Start, 1973; Gardeström, 1987). A problem with PEPCK acting as a carboxylase remains – that of its decarboxylase activity. The only known mechanism by which the carboxylase activity of PEPCK could be enhanced compared with its decarboxylase activity is by changes in concentration of its substrates and potentially pH. In the cytosol of many mammalian cells, flowering plant cells and bacteria, the concentration of OAA is very low – of the order of 20 μM (see Titheradge *et al*., 1992; Agostino *et al*. 1996; Petersen *et al*., 2001). At this concentration, changes in OAA concentration would have a very large effect on the decarboxylase activity of PEPCK (fig. 3 in Walker *et al*., 2002). A substrate cycle might exist between the carboxylase and decarboxylase activities, and a small decrease in OAA concentration could potentially bias the reaction in favour of carboxylation. Support for this is provided by the observation that feeding malate or aspartate to *L. hyperborea* tissue discs increases the intracellular pool of dicarboxylic acids, and under these conditions PEPCK was proposed to decarboxylate OAA (Weidner and Küppers, 1982). If the supply of inorganic carbon to brown algae is restricted, photosynthesis is maintained, suggesting the provision of CO_2 from an internal store (Schmid and Dring, 1986a,b). This CO_2 could potentially arise by changes in the cytosolic concentration of OAA that bias the PEPCK reaction in favour of decarboxylation. This change could be brought about by an efflux of malate or aspartate from the vacuole and/or an increase in NAD/NADH ratio in the cytosol which would alter the equilibrium position of malate dehydrogenase in favour of OAA (this is because of the equilibrium position of the malate dehydrogenase reaction [malate] × [NAD] = constant × [OAA] × [NADH]). Similarly, the enhancement of photosynthesis by blue light (Schmid and Dring, 1986a,b) could be explained in the same way. The physiological relevance of this mechanism would be to assist in the acquisition of inorganic carbon from the sea, and to provide a constant high supply of CO_2 for photosynthesis. The reason why PEPCK might be used could be related to the fact that a futile cycle using just PEPCK does not consume ATP, but that one using PEPC/PEPCK would. This might be important in algae, because for short or long periods of time they live under conditions in which photosynthesis is less than respiration. Another potential advantage of this mechanism is that rapid changes in the direction of net flux would be possible.

Concluding remarks regarding PEPCK in algae

The metabolism of algae has, in general, been much less studied than that of flowering plants, and is consequently much less understood. In addition, the

algae represent a diverse phylogenetic group and are likely to show significant differences in their metabolism. As pointed out by Kerby and Raven (1985), it is essential to establish which enzymes, capable of carboxylation of PEP/pyruvate and decarboxylation of OAA/malate, are present in the different groups of algae. It is also important to establish under what conditions these enzymes are present and how abundant they are. Towards this end it is important to study this in cultures of microscopic algae under a range of conditions.

D. SITUATIONS IN WHICH LARGE AMOUNTS OF AMINO ACIDS ARE IMPORTED

Under certain conditions in many unicellular organisms and certain cell types in multicellular organisms PEPCK can be viewed as having a role in amino acid metabolism. In these, large amounts of amino acids are imported, and often they are deaminated and a proportion of the resulting carbon skeletons is converted to PEP and used in gluconeogenesis, or is converted to pyruvate and is used by the Krebs cycle, although there are other possible fates for PEP and pyruvate. In many of these organisms and cells PEPCK is utilised to convert OAA to PEP. In mammals, such amino acid metabolism occurs in the proximal tubule of the kidney, in which it is a component of a mechanism involved in the regulation of body pH. In plants, PEPCK is likely to serve a similar function in many sink tissues, such as developing seeds, roots, flowers, some parasitic plants and transport tissues.

1. Kinetoplastids

These are unicellular organisms and different species are responsible for a number of important diseases, such as sleeping sickness (see Alexander, 1979 for introductory account). For this reason they have been the subject of much study, and for this purpose they are often grown in culture. These organisms often spend their life cycle in two hosts. For example, *Trypanosoma brucei*, the cause of sleeping sickness, spends part of its life in the blood plasma of humans or antelopes and the other part in the tsetse fly. During feeding on blood, the fly ingests the parasite, which multiplies in its gut and then moves to the salivary gland. Another cycle of infection begins when the fly bites a mammalian host. However, it should be noted that there are differences between protozoan genera and species, for example, *Leishmania* (see Mottram and Coombs, 1985). The trypanosomes are polymorphic, i. e. their structure is different in the vertebrate and the insect and their metabolism is very different. It may be somewhat surprising that trypanosomes contain PEPCK–ATP and not PEPCK–GTP. However, over recent years these organisms have been found to possess a number of plant-like features, such as the alternative oxidase which transfers electrons from ubiquinol to oxygen and hence can act to bypass cytochrome oxidase in the mitochondrial electron transfer chain. In addition, the primary pump at the plasma membrane is an ATP-driven H^+ pump (Sakano, 2001, and references therein). Trypanosomes are thought to be closely related to the euglenids

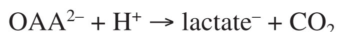
(Margulis and Schwartz, 1988). The evolutionary events leading to modern protoctists, such as trypanosomes and *Euglena* are complex, and many different endosymbiotic events have occurred. The reader is referred to Margulis and Schwartz (1998) for a discussion of this topic.

In trypanosomes PEPCK is present in an organelle called the glycosome. In the blood form of *T. brucei* the glycosome contains glycolytic enzymes; however, in the insect form the glycosome enzyme complement changes dramatically, and large amounts of PEPCK appear (Kueng *et al*., 1989). In trypanosomes PEPCK is very abundant, for example, in *T. brucei* it comprises about 0.8% of total protein (Hunt and Köhler, 1995), which is similar to that in the leaf of a C4 plant (Walker *et al*., 2002). The bloodstream form of PEPCK in *T. brucei* depends entirely on glycolysis for energy production and excretes large amounts of pyruvate. Upon entering the insect, PEPCK and malate dehydrogenase increase 10- and 100-fold respectively, and hexokinase and pyruvate kinase fall 15- and 20-fold respectively (Hunt and Köhler, 1995 and references therein). Although it should be noted that in *T. cruzi* PEPCK is always present (Linss *et al*., 1993). At the same time a fully functional Krebs cycle and cytochrome-mediated electron-transport chain develop. Although in cultured *T. cruzi* PEPCK can act as a carboxylase, this is likely to be a result of very high unphysiological concentrations of sugars, and is likely to act as a decarboxylase *in vivo* (Urbina, 1987). In the insect gut these organisms are thought to live solely on an amino acid diet (Urbina, 1987). After uptake, amino acids are deaminated. One enzyme known to be important in this is glutamate dehydrogenase. The α-ketoglutarate produced is used by the Krebs cycle. A proportion of the OAA so produced leaves the Krebs cycle and is converted to PEP by PEPCK. PEP can then be used in gluconeogenesis or converted to pyruvate to replenish the Krebs cycle (for reviews see Cazzulo, 1994; Urbina, 1994). Therefore, the metabolic function of PEPCK in trypanosomes, under certain circumstances, is somewhat similar to that in acidotic kidney and that proposed for maize roots assimilating ammonium.

2. Cells in Multicellular Organisms: Lymphocytes, Kidney, Roots, the Transport System of Plants and Developing Sinks

In multicellular organisms PEPCK is often present in cell types that import large amounts of amino acids. A number of these are discussed in section IX.F regulation of pH. PEPCK–GTP is present in lymphocytes in which there are about 0.7 units of activity per gram fresh weight (one unit = 1 μmol min^{-1} of substrate converted to product at 25°C, and under other conditions in *in vitro* assay in which maximum activity is measured, Newsholme *et al*., 1985). Glutamine is essential for cultured lymphocytes, and is utilised at a rate four-fold higher than glucose. Glutamine is utilised by the glutaminolysis pathway, the initial step involving the deamination of glutamine by glutaminase, to produce NH_4^+ and glutamate. Glutamate is transaminated by aspartate aminotransferase, and the α-ketoglutarate so produced enters the Krebs cycle and is metabolised to OAA.

OAA is then used as the substrate for aspartate aminotransferase; hence the pathway is a cycle (for the metabolic scheme see Newsholme *et al.*, 1985). The main end-products of the pathway that accumulate are aspartate and ammonium. However, a smaller amount of lactate accumulates and this involves PEPCK. The functional significance of the accumulation of lactate is unclear. One possibility that we feel is worthy of consideration is as follows. Overall the metabolic pathway proposed by Newsholme *et al.* (1985) is proton neutral if intermediates are not withdrawn from the Krebs cycle. However, if some of the NH_4^+ produced by the action of glutaminase leaves the cell as NH_3, then this leaves a proton within the cell. This process could potentially lead to intracellular acidification. This is similar to the situation in kidney during acidosis. The action of PEPCK in catalysing the formation of lactate effectively consumes a proton. Overall the reaction can be represented by:



$$OAA^{2-} + H^+ \rightarrow lactate^- + CO_2$$

The other route of lactate formation in these cells, using malic enzyme (Newsholme *et al.*, 1985), would have the same effect.

$$malate^{2-} + H^+ \rightarrow lactate^- + CO_2$$

The interconversion of malate and OAA is proton neutral if the NADH formed is used by oxidative phosphorylation. How metabolic processes consume or produce protons is discussed in detail in section IX.F.

E. ANAEROBIC METABOLISM

Early in the evolution of life, the Earth's atmosphere did not contain oxygen (see Stanley, 1998), and organisms obtained energy by substrate-level phosphorylation, using reactions like that catalysed by PEPCK, and not oxidative phosphorylation (see Hochachka and Mustafa, 1972; Margulis and Schwartz, 1998). Even today, many animals are surprisingly tolerant of a lack of oxygen; for example, some freshwater turtles can live for some months and goldfish for several days without oxygen (for an overview see Hochachka, 1982). The metabolism of these animals possesses some features in common with the roots of flowering plants subjected to anaerobic conditions; for example, both roots and goldfish produce large amounts of ethanol (cf. Hochachka, 1982; Dennis *et al.*, 1997). Other animals are even more adept at coping with anaerobiosis, and many of them utilise PEPCK as a carboxylase. Some helminths that live in the gut of mammals live their entire life without the need for oxygen (for a review see Saz, 1981). In invertebrates specialised to live under anaerobic conditions, there exist a wide range of these fermentation pathways, many of which utilise PEPCK (see Hochachka, 1982). The purpose of these fermentations is to produce ATP in the absence of oxygen, while avoiding an overreduction of the NAD(P) pool. In these anaerobic organisms the abundance of PEPCK is high,

for example, in the cytoplasm of the gut worm *A. suum* it is about one part in 50 of total protein (Rohrer *et al.*, 1986). Some molluscs, such as the oyster and other intertidal bivalves, are facultative anaerobes. The muscle of these stores large amounts of glycogen which, under aerobic conditions, is metabolised to pyruvate, which is then utilised by the Krebs cycle. This is similar to the red muscle of vertebrates (see Newsholme and Start, 1973). In general, red muscle uses aerobic metabolism, but white muscle (such as that common in chicken and fish) uses anaerobic metabolism. However, in intertidal bivalves under anaerobic conditions PEP is converted to OAA by PEPCK, and subsequently a number of reactions occur, leading to the accumulation of succinate and alanine. In vertebrates, white muscle often metabolises glucose anaerobically to lactate in order to obtain ATP, this lactate is transferred by the blood to the liver and kidney and used in gluconeogenesis – a process that utilises PEPCK. This is known as the Cori cycle. In addition to being a component of the pathway of gluconeogenesis, that is, the conversion of lactate to glucose, PEPCK also functions in pH homeostasis because the synthesis of lactate produces protons, whereas its conversion to glucose consumes protons. Overall, the Cori cycle, of which PEPCK is a component, facilitates anaerobic metabolism in muscle. Some accounts say that lactate synthesis does not produce protons; however, this is because the hydrolysis of the ATP so produced is not considered (see Sakano, 2001). In some vertebrates, certain muscle types contain PEPCK, and in these a proportion of lactate is converted to sugar within the muscle (Hochachka, 1982 and references therein).

In flowering plants PEPCK has not been found to be induced in their roots by anaerobiosis (Smith, 1985; Walker *et al.*, 2001). However, this does not rule out the possibility that PEPCK plays a role in anaerobiosis, in the roots of some species (under certain circumstances) or in some other tissues. In the roots of flowering plants under anaerobic conditions, the accumulation of lactate appears to play a transitory role and there is no mechanism resembling the Cori cycle (Dennis *et al.*, 1997). Instead, a key feature of the response of the roots of flowering plants is, like the goldfish (Hochachka, 1982), to produce ethanol (Dennis *et al.*, 1997). A feature of anaerobiosis in many organisms is that it produces intracellular acidification (see Hochachka and Mustafa, 1972; Kurkdjian and Guern, 1989; Sakano, 2001). A common feature of plant roots subjected to anaerobiosis is the consumption of their intracellular malate pool (Smith and ap Rees, 1979; Sakano, 2001). This is often mediated by malic enzyme, and ethanol is produced; this acts to consume protons (Sakano, 2001 and references therein).

$$\text{malate}^{2-} + 2\text{H}^+ \rightarrow \text{ethanol} + 2\text{CO}_2$$

It is possible that flowering plants do employ a cycle between roots under anaerobiosis and the shoot and import malate to alleviate intracellular acidification. The resulting acidification in the leaf could be overcome by increasing the rate of assimilation of stored nitrate from vacuoles in the cells of the shoot. Nitrate

assimilation is a proton-consuming process. This would be similar to the cycling of malate to the root that occurs during nitrate assimilation under non-anaerobic conditions (Raven and Smith, 1976).

In micro-organisms a diverse range of fermentations occur under anaerobic conditions. These fermentation pathways are organised to generate ATP by substrate-level phosphorylation, and to avoid over-reducing the cells' NAD(P) pool. Such fermentation pathways utilising PEPCK as a carboxylase are likely to be widespread, and occur, for example, in cultured *Euglena* (see Pönsgen-Schmidt *et al*., 1988), kinetoplastids (see Urbina, 1987), bacteria (Macy *et al*., 1978; Kapke *et al*., 1980; Schobert and Bowien, 1984; Samuelov *et al*., 1991; Podkovyrov and Zeikus, 1993; Schöcke and Weimer, 1997) and chytrids (Reymond *et al*., 1992). In these organisms PEPCK is often very abundant: about 1 part in 100 of total protein (see Pönsgen-Schmidt *et al*., 1988; Hunt and Köhler, 1995).

PEPCK catalyses a reversible reaction, so this raises the question as to how the direction of the reaction is determined *in vivo*. It has long been thought that PEPCK acts as a decarboxylase in most tissues *in vivo* because its affinity for CO_2 is low compared with the concentration of CO_2 within the cell (Utter and Kolenbrander, 1972). It was suggested that one reason that PEPCK may act as a carboxylase in the gut worm *A. suum* is that the concentration of CO_2 in the mammalian gut is high (Rohrer *et al*., 1986). However, recent work suggests that at least for the enzyme from flowering plants this low affinity is a result of unphysiological *in vitro* assay conditions (Chen *et al*., 2002). It is possible that the enzyme from obligate anaerobes possesses properties that enhance its carboxylase activity *in vivo*. It has been reported that the enzyme from *A. suum* has a low affinity for OAA (see Saz, 1981). Rohrer *et al*. (1986) suggested that in *A. suum* the direction of the reaction is likely to be controlled to a large extent by concentrations of substrates and potentially ATP. A similar conclusion was reached for kinetoplastids. Studies using strains of *C. glutamicum* possessing different amounts of PEPCK have shown that the intracellular concentration of OAA is the critical factor in controlling flux through the decarboxylase reaction of PEPCK (Petersen *et al*., 2001). This suggests that an important factor in allowing PEPCK to act as a carboxylase *in vivo* is the maintenance of a low concentration of OAA. In many situations OAA and malate are interconverted by malate dehydrogenase and the equilibrium position of this enzyme is greatly in favour of malate. This maintains a low concentration of OAA in the cell. However, because this reaction also utilises NAD(H), the concentration of these will alter the concentration of OAA (for a discussion see Newsholme and Start, 1973). The concentration of OAA is proportional to NAD/NADH – this can be seen by just rearranging the equation for the equilibrium constant of this reaction. In roots subjected to anaerobiosis the NADH concentration increases greatly (Dennis *et al*., 1997). This would suggest that OAA concentration would decrease and consequently greatly inhibit the decarboxylase activity of PEPCK (fig. 3 in Walker *et al*., 2002). Another factor that might be important in enhancing the carboxylase activity is the lowering of cytosolic pH (see Hochachka and Mustafa,

1972; Sakano, 2001). This might enhance the carboxylase activity of PEPCK, because the pH optimum of the carboxylase activity of PEPCK is lower than that of the decarboxylase activity (Hochachka and Mustafa, 1972; Utter and Kolenbrander, 1972). Similarly, Podestra and Plaxton (1991) attributed an activation of cytosolic pyruvate kinase in germinating castor oil seeds to an anoxia-induced cytosolic acidification. An increase in the pH of the stroma of the chloroplast upon illumination is important in the activation of several enzymes of the Benson–Calvin cycle (see Plaxton, 1997). Another factor that would enhance the carboxylase activity is a decrease in the ATP:ADP ratio which might occur under anaerobiosis (Dennis *et al*., 1997). A high ATP:ADP ratio markedly inhibits the carboxylase activty of PEPCK–ATP (fig. 6 in Urbina, 1987; fig. 3 in Walker *et al*., 2002), ATP is also an inhibitor of PEPCK–GTP with a K_i of 0.5 mM (Rohrer *et al*., 1986). This leads us to the question of why PEPCK is not utilised in the roots of flowering plants during anaerobiosis. This tissue appears to lack the ability to perform complex fermentations and therefore OAA is not synthesised. In fact OAA/malate is decarboxylated in order to consume protons (Sakano, 2001). Malic enzyme is often used in the dissimilation of malate (Sakano, 2001); one factor contributing to why PEPCK is not utilised in this situation may be that the PEPCK reaction could be biased towards carboxylation.

F. PEPCK AND THE METABOLIC REGULATION OF pH

1. Distribution of PEPCK in Flowering Plants

In the 1950s it was reported that PEPCK was present in a wide range of flowering plant tissues (Mazelis and Vennesland, 1957). However, until recently it was generally thought that PEPCK was only important in the metabolism of a few tissues from flowering plants, namely leaves of C4 and CAM plants, storage tissues of germinating seeds and some fruit. However, recent work has clearly established that PEPCK is present, often in large amounts, in many parts of flowering plants such as the roots, stems, fruit, developing seeds and leaves of C3 plants (Kim and Smith, 1994; Walker *et al.*, 1999; Chen *et al*., 2000; Lea *et al*., 2000). Localisation of PEPCK protein by immunohistochemistry and PEPCK mRNA by *in situ* hybridisation, showed that PEPCK was not present throughout structures such as seeds and roots, but was localised in certain types of cell. Generally these were heterotrophic cells that were likely to have a high metabolic rate, such as phloem, xylem parenchyma, pericycle and trichomes (Walker *et al*., 1999; Chen *et al*., 2000). These cells are all sinks, which raised the possibility that PEPCK played a role in the metabolism of imported assimilates such as nitrogen-containing compounds.

2. The Abundance of PEPCK in Many Plant Tissues is Increased by Treatments that Lower Cytosolic pH

Maize roots were used as a model sink tissue for nitrogen to investigate the function of PEPCK further. Maize roots were used because their metabolism is

easily manipulated by feeding different nitrogenous compounds, and plant material is easy to obtain. In addition, certain relevant aspects of their biology had been studied, such as their structure in relation to transport processes (Zimmermann *et al*., 2000 and references therein), and some effects of feeding nitrogenous compounds (Gerendás *et al*., 1990; Gerendás and Ratcliffe, 2000). In maize, and a number of other plants that were fed different forms of nitrogen to their roots, such as nitrate, ammonium, urea or different amino acids, PEPCK was usually only present in roots of plants fed NH_4^+/ NH_3. Feeding maize roots NH_4^+/ NH_3 increased the abundance of PEPCK from undetectable amounts to high activity (between 0.3 and 1.0 μmol min^{-1} g (fresh wt)$^{-1}$, depending on other conditions) (Walker *et al*., 2001). However, because PEPCK is localised in a certain type of cell, in these the activity was very high (Walker *et al*., 2001). This suggested that, in maize roots, the presence of PEPCK was dependent on the specific presence of NH_4^+/ NH_3 and not nitrogen-containing compounds in general. This raised the question as to why feeding NH_4^+/ NH_3 induced PEPCK? When NH_4^+/ NH_3 was fed to maize roots in the presence of buffer, PEPCK was only present when the pH of the buffer was below approx. 5.0. The significance of this observation is that feeding ammonium to maize roots at low external pH has been shown to lower the cytosolic pH of maize roots; this was most pronounced in root tips (Gerendás *et al*., 1990). It should be noted that the experiments of Gerendás *et al*., (1990) differed somewhat from those of Walker *et al*. (2001) in that the plant material was younger, and with the NMR technique used it was not possible to determine the change in cytosolic pH of different types of cell within the root. In contrast, feeding NH_4^+/ NH_3 at higher pH raises cytosolic pH (Gerendás and Ratcliffe, 2000). The pH of the feeding solution alters the equilibrium between NH_4^+ and $NH_3 + H^+$ (pK 9.25, in this case pK is equivalent to the pH at which NH_4^+ is 50% dissociated to $NH_3 + H^+$; for a discussion of equilibria, see Jones and Atkins, 1999), the lower the pH the greater the proportion of NH_4^+. When the pH of the feeding solution is low, NH_3 is at lower abundance, and therefore less enters root cells by passive diffusion. Uptake of NH_4^+ can lower cytosolic pH in two ways, firstly, a small proportion of NH_4^+ will release a proton on entering the cytosol in order to maintain the equilibrium between NH_4^+ and $NH_3 + H^+$, secondly, its assimilation into amino acids is a proton-producing process (see, Gerendás and Ratcliffe, 2000). This is because the synthesis of α-ketoglutarate from glucose, required for incorporation of NH_4^+ into glutamate by the action of glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle, is a proton-producing process. Feeding NH_4^+/NH_3 at higher pH increases cytosolic pH (Gerendás and Ratcliffe, 2000). This is because at higher pH NH_3 is more abundant in the buffer and a greater proportion of NH_4^+/NH_3 enters root cells by passive diffusion of NH_3 across the plasma membrane. On entering the cytosol a high proportion of NH_3, accepts a proton in order to maintain the equilibrium between NH_4^+ and $NH_3 + H^+$ (Gerendás and Ratcliffe, 2000). It should be noted that if NH_3 is taken up by a cell, a much greater proportion of it accepts a proton than would be released from NH_4^+ if that were taken up. This is because the pK is 9.25 and, therefore, at the pH of the cytosol,

about 7.3, most should be as NH_4^+ ; examination of a plot of the abundance of NH_3 and NH_4^+ against pH illustrates this point. Co-feeding ammonium and nitrate at low pH decreased or abolished the induction of PEPCK, this being dependent on their concentrations. Nitrate assimilation will counteract acidosis because its assimilation is a proton-consuming process (Raven and Smith, 1976).

These observations raised the question as to whether other treatments that are known to lower cytosolic pH increase the abundance of PEPCK. Presenting butyric acid to roots also induced PEPCK, in this case the appearance of PEPCK was quicker and the final amount present was about half as much (Walker *et al.*, 2001). Uptake of the undissociated form of butyric acid results in its dissociation on entering the cytosol, in order to maintain the equilibrium between undissociated acid/dissociated acid plus proton (Kurkdjian and Guern, 1989). Growing plants in an atmosphere of 5% CO_2 for 5 days, which causes intracellular acidification of leaves (Pfanz and Heber, 1986), also induced PEPCK in their leaves, but little change was observed in their roots (Walker *et al.*, 2001). The amount of PEPCK induced by this treatment was about 20% of that induced in maize roots by feeding ammonium. In the soil, CO_2 concentrations are often at 5%, whereas the atmospheric CO_2 level is 0.03%. The CO_2 decreases pH because it reacts with water to release a proton ($CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$). However, this is complicated by the fact that this reaction is slow and in living organisms the enzyme carbonic anhydrase speeds up the reaction by a factor of 10^7 (Stryer, 1981); therefore, the acidifying effect of CO_2 is also dependent on the presence of this enzyme. The abundance of carbonic anhydrase in different tissues and compartments within a cell is highly regulated (Burnell, 1990). In certain tissues of the kidney of some species, PEPCK is induced in response to acidosis (Hanson and Patel, 1994). In kidney, PEPCK is a component of a mechanism used to regulate body pH, which as is shown later, is metabolically similar to that likely to occur in maize roots. Having found that – in a range of tissues – the abundance of PEPCK is greatly increased by treatments known to lower cytosolic pH, it was necessary to consider what function PEPCK might have in this situation.

3. *An Overview of pH Homeostasis in Plants*

Before considering what function PEPCK might have in acidosis, it is necessary to understand what is known about how perturbations in intracellular pH are prevented in flowering plants. In living things a number of metabolic processes are neutral with respect to the production of protons. For example, the utilisation of glucose by glycolysis, the Krebs cycle, the use of NADH by oxidative phosphorylation, and the subsequent hydrolysis of the ATP formed, are neutral with respect to the production of protons (Stryer, 1981). However, if intermediates are drained from the Krebs cycle then the process may no longer be neutral. For example, the use of α-ketoglutarate produced by the Krebs cycle in the assimilation of ammonium into glutamate produces protons. Similarly, the import and subsequent metabolism of some compounds such as amino acids

is often not neutral. There are several reasons why it is important to maintain neutrality – one being that protons disrupt the conformation and structure of proteins, and another being that the difference in proton concentration across a membrane is a form of energy which is harnessed to drive a number of processes. A discussion of this topic is beyond the scope of this article. The concentration of protons within the cell is very low, at pH 7 their concentration is 0.1 μM, at pH 6 it is 1 μM, and at pH 5 it is 10 μM. This follows from the definition of pH, pH = –log[H^+]. However it should be emphasised that protons are highly reactive and do not exist in the free state, but form hydronium ions H_3O^+; these attract a water molecule to each hydrogen to form the ion which is actually present ($H_9O_4^+$) (see Jones and Atkins, 1999). For simplicity, the shorter formula, H^+, or proton, will be used. A number of mechanisms are used to maintain the required proton concentration – the mechanism(s) used depends on the tissue and the cause, strength and duration of the perturbation. Firstly, the buffering capacity of the cellular constituents, HPO_4^{2-} being particularly important (Netting, 2002), tends to counteract these perturbations. However, this mechanism has little capacity and is soon overwhelmed (Kurkdjian and Guern, 1989 and references therein). Secondly, protons may be transferred to the vacuole. However, the vacuole possesses a limited buffering capacity, which is aggravated by the fact that the synthesis of metabolites such as malate, which act as buffers in the vacuole, produce as many protons as their dissimilation consumes (Smith and Raven, 1979). Plant cells can accumulate large amounts of protons in the vacuole as in fruit and leaves of CAM plants, but this is only possible because malate or citrate, whose synthesis gives rise to these protons, buffer them. Thirdly, protons may be transferred across the plasma membrane into the apoplast. In animals a number of membrane transport systems effectively transfer protons between the cell and the extracellular fluid (Alper, 1991). In vertebrates, excess protons are transported by the circulatory system, and in the structure, such as the lung or gill used for gas exchange, combine with bicarbonate to form CO_2, which is then removed from the body. However, this process only effectively removes CO_2 from the animal, it does not remove protons produced in excess of CO_2. During periods of acidosis, excess protons are removed from the blood by the kidney in many vertebrates, a process that in many species, as explained later, utilises PEPCK. Similarly, in plants a number of proteins in the plasma membrane also have the overall effect of transporting protons between the cytosol and apoplast (the intercellular spaces between plant cells) (Kurkdjian and Guern, 1989 and references therein); however, the apoplast has a limited buffering capacity, and in the long term this is not a solution to preventing perturbations of pH, because protons cannot be removed from the apoplast of most tissues of land plants. An important exception is the root, because protons can often diffuse from it to the soil. An interesting consequence of this is that plants grown on nitrate often make the soil alkaline, whereas those grown on ammonium make it acid (Raven and Smith, 1976 and references therein). The root is therefore, in some ways analogous to the kidney of animals. Plants are able to transport protons between tissues,

because the synthesis of organic acids in one tissue and their dissimilation in another effectively transports protons or hydroxyl groups between tissues.

This process is intimately linked to the assimilation of nitrate and ammonium, because these processes are not proton neutral, and in many situations are the major factor resulting in pH imbalance in plants (for a review, see Raven and Smith, 1976). An example of this is plants assimilating nitrate in their leaves. In this case, malic acid is synthesised in the leaf – a process that produces protons. Malate is then transported to the root in the phloem, and the protons produced in its synthesis remain in the leaf. In the root malate is dissimilated – a process which consumes protons. The resulting increase in pH is offset by transport processes at the plasma membrane of root cells, which effectively transfer hydroxyl groups to the soil (Touraine *et al*., 1992).

4. How Do Metabolic Processes Consume or Produce Protons?

In the late 1940s and early 1950s an important part of the answer to the enzymatic basis of the long-known observation that plants accumulate and subsequently dissimilate organic acids was found, with the discovery of the enzymes PEPCK, malic enzyme and PEPC (for reviews, see Vennesland and Conn, 1952; Utter and Kolenbrander, 1972). A large number of studies showed that the synthesis and dissimilation of organic acids was widespread in plants, and was modulated by various environmental factors. Davies (1973, 1986) published the biochemical pH-stat model, according to which, increases in pH were opposed by the synthesis of malic acid – a process which utilises PEPC and produces protons. Decreases in pH were opposed by the dissimilation of malic acid by malic enzyme, which consumes protons. At this time, the fact that PEPCK is widely distributed in flowering plants was not appreciated. It now seems likely that PEPC is responsible for the net synthesis of malate in many tissues of flowering plants. However, although in some situations the malic enzymes are likely to function in dissimilation, in others PEPCK is likely to serve this function. The biochemical pH stat was refined by Sakano (1998), and it was pointed out that the explanation of how PEPC generates protons or how malic enzyme consumes them was incorrect and what must be considered is the proton production or consumption associated with the production of substrates and consumption of products of the PEPC and malic enzyme reactions. This is quite a complex topic, because a number of factors must be considered; however it is a key concept in understanding pH homeostasis.

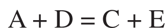
For example, if the first reaction in the pathway is:

$$A = B + C$$

The second reaction is:

$$B + D = E$$

The sum of the reactions is:



$$A + D = C + E$$

This process is carried out for each reaction of the pathway. However, there are a number of points to bear in mind.

1. The ionisation state of the carboxylic acid and amino group of amino acids – at the pH of the cytosol these are almost completely ionised. An inspection of their p*K* values illustrates this point (many p*K* values are given in Voet and Voet, 1995). It should be noted that malate refers to the 'dissociated acid', not malic acid. Malate is the conjugate base of the weak acid malic acid. At the pH of the cytosol (about 7.3) malic acid is almost fully dissociated (the p*K* values for the two carboxylic acid groups of malic acid are pK_1, 3.40; pK_2, 5.26). Caution should be exercised because many textbooks and research papers show the wrong ionisation states of metabolites and the incorrect overall equation for a reaction or a pathway. The account of Stryer (1981), as far as we aware, is correct, and the reader is referred to this account for structural formulae, reactions catalysed by individual enzymes, and metabolic pathways.
2. If NADH is a product then this will consume a proton if it used by oxidative phosphorylation.

$$NADH + \tfrac{1}{2}O_2 + H^+ \rightarrow NAD^+ + H_2O$$

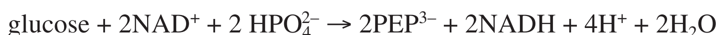
3. If ATP is a product then its subsequent fate must be considered. If it is hydrolysed then

$$ATP^{4-} + H_2O \xleftrightarrow{\text{ATPase}} ADP^{3-} + HPO_4^{2-} + H^+$$

This equation also shows that the synthesis of ATP by oxidative phosphorylation is a proton-consuming process. For a description of the ionisation state of the phosphate ion at different pH values the reader is referred to Jones and Atkins (1999).

4. A good check that an equation is correct is that the charge and the number of atoms or ions of each element should be the same on both side of the equation. In addition, in general the synthesis of a carboxyl group of a carboxylic acid produces a proton and its degradation removes a proton.

Production of malate from glucose using PEPCK.
Production of PEP from glucose:



$$glucose + 2NAD^+ + 2\,HPO_4^{2-} \rightarrow 2PEP^{3-} + 2NADH + 4H^+ + 2H_2O$$

PEPCK:

$$PEP^{3-} + CO_2 + ADP^{3-} \rightarrow OAA^{2-} + ATP^{4-}$$

Hydrolysis of ATP:

$$ATP^{4-} + H_2O \leftrightarrow ADP^{3-} + HPO_4^{2-} + H^+$$

Malate dehydrogenase:

$$OAA^{2-} + NADH + H^+ \rightarrow malate^{2-} + NAD^+$$

Sum of reactions:

$$\text{glucose} + 2CO_2 \rightarrow 2\text{malate}^{2-} + 4H^+$$

A similar calculation shows that synthesis of malate by PEPC is equivalent (Sakano, 1998). This is important because it shows that the synthesis of malate from glucose using PEPC or PEPCK is equivalent in terms of proton production. This suggests that PEPCK cannot prevent a fall in intracellular pH in maize root by replacing PEPC as the carboxylase used in the synthesis of malate from glucose.

The next step in the analysis is to consider whether the decarboxylase activity of PEPCK potentially could play a role in preventing a decrease in intracellular pH.

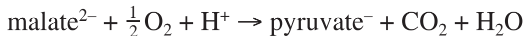
Synthesis of pyruvate from malate using malic enzyme.
Malic enzyme:

$$\text{malate}^{2-} + NAD(P)^+ \rightarrow \text{pyruvate}^- + NAD(P)H + CO_2$$

Oxidative phosphorylation:

$$NADH + \tfrac{1}{2}O_2 + H^+ \rightarrow NAD^+ + H_2O$$

Sum of reactions:



$$\text{malate}^{2-} + \tfrac{1}{2}O_2 + H^+ \rightarrow \text{pyruvate}^- + CO_2 + H_2O$$

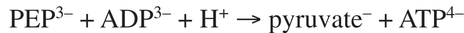
Synthesis of pyruvate from malate using PEPCK.
Malate dehydrogenase:

$$\text{malate}^{2-} + NAD^+ \rightarrow OAA^{2-} + NADH + H^+$$

PEPCK:

$$OAA^{2-} + ATP^{4-} \rightarrow PEP^{3-} + CO_2 + ADP^{3-}$$

Pyruvate kinase:

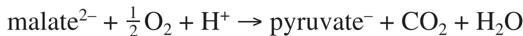


$$PEP^{3-} + ADP^{3-} + H^+ \rightarrow \text{pyruvate}^- + ATP^{4-}$$

If NADH is used by oxidative phosphorylation:

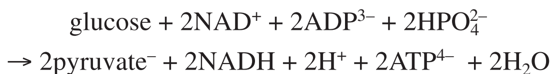
$$NADH + \tfrac{1}{2}O_2 + H^+ \rightarrow NAD^+ + H_2O$$

Sum of reactions:



$$\text{malate}^{2-} + \tfrac{1}{2}O_2 + H^+ \rightarrow \text{pyruvate}^- + CO_2 + H_2O$$

Synthesis of pyruvate from glucose.
The equation for glycolysis can be written as:



$$\text{glucose} + 2NAD^+ + 2ADP^{3-} + 2HPO_4^{2-}$$
$$\rightarrow 2\text{pyruvate}^- + 2NADH + 2H^+ + 2ATP^{4-} + 2H_2O$$

If ATP is hydrolysed:

$$ATP^{4-} + H_2O \rightarrow ADP^{3-} + HPO_4^{2-} + H^+$$

then the equation becomes:

$$\text{glucose} + 2\text{NAD}^+ \rightarrow 2\text{pyruvate}^- + 2\text{NADH} + 4\text{H}^+$$

If NADH is used by oxidative phosphorylation:

$$\text{NADH} + \tfrac{1}{2}\text{O}_2 + \text{H}^+ \rightarrow \text{NAD}^+ + \text{H}_2\text{O}$$

then the equation becomes:

$$\text{glucose} + \text{O}_2 \rightarrow 2\text{pyruvate}^- + 2\text{H}^+ + 2\text{H}_2\text{O}$$

Therefore, the production of pyruvate from malate using either PEPCK or malic enzyme are equivalent in terms of proton production, and produce two less protons than the synthesis of pyruvate from glucose. However, if either malic enzyme or PEPCK and associated metabolism are to consume protons in the long term, then it is essential that the malate which gives rise to OAA, or in some cases amino acids whose metabolism gives rise to OAA, is imported into the cell. This is because the synthesis of malate or OAA from glucose produces two protons.

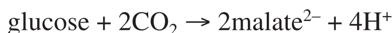
The next step in the analysis is to ask why maize roots fed ammonium need to alleviate problems arising from an excess production of protons. The GS/GOGAT cycle incorporates NH_4^+ into amino acids in maize roots, this requires α-ketoglutarate for the reaction catalysed by GOGAT. Alpha-ketoglutarate is likely to be synthesised by the Krebs cycle, which requires the input of pyruvate and malate.

How many protons are produced in the synthesis of α-ketoglutarate from glucose?

Synthesis of pyruvate from glucose:

$$\text{glucose} + \text{O}_2 \rightarrow 2\text{pyruvate}^- + 2\text{H}^+ + 2\text{H}_2\text{O}$$

Synthesis of malate from glucose:



$$\text{glucose} + 2\text{CO}_2 \rightarrow 2\text{malate}^{2-} + 4\text{H}^+$$

The synthesis of α-ketoglutarate from pyruvate and malate by the Krebs cycle results in two protons being produced for each α-ketoglutarate synthesised:

$$\text{glucose} + 2\text{O}_2 \rightarrow \alpha\text{-ketoglutarate}^{2-} + \text{CO}_2 + 3\text{H}_2\text{O} + 2\text{H}^+$$

The next step is to consider how many protons are produced in the assimilation of NH_4^+ by GS/GOGAT.

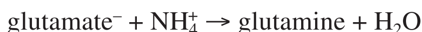
GS:

$$\text{glutamate}^- + \text{NH}_4^+ + \text{ATP}^{4-} \rightarrow \text{glutamine} + \text{ADP}^{3-} + \text{HPO}_4^{2-} + \text{H}^+$$

Resynthesis of ATP by oxidative phosphorylation:

$$\text{ADP}^{3-} + \text{HPO}_4^{2-} + \text{H}^+ \rightarrow \text{ATP}^{4-} + \text{H}_2\text{O}$$

Sum of reactions:

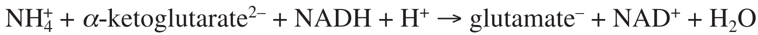


$$\text{glutamate}^- + \text{NH}_4^+ \rightarrow \text{glutamine} + \text{H}_2\text{O}$$

GOGAT:

$$\alpha\text{-ketoglutarate}^{2-} + \text{glutamine} + \text{NADH} + \text{H}^{+} \rightarrow 2\text{glutamate}^{-} + \text{NAD}^{+}$$

Sum of reactions:



$$\text{NH}_4^{+} + \alpha\text{-ketoglutarate}^{2-} + \text{NADH} + \text{H}^{+} \rightarrow \text{glutamate}^{-} + \text{NAD}^{+} + \text{H}_2\text{O}$$

Synthesis of glutamate from glucose and ammonium:

$$\text{glucose} + \text{NH}_4^{+} + \tfrac{3}{2}\text{O}_2 \rightarrow \text{glutamate}^{-} + \text{CO}_2 + 3\text{H}_2\text{O} + 2\text{H}^{+}$$

Therefore the synthesis of one glutamate from NH_4^+ and glucose produces two protons. This is in agreement with Gerendás and Ratcliffe (2000). However, if either malate is imported into the tissue and used either directly or after conversion to pyruvate by the Krebs cycle, then no protons are produced. This is a key point in understanding the function of PEPCK in pH regulation in maize roots fed ammonium.

5. How Might PEPCK Function in pH Homeostasis in Maize Roots Fed Ammonium?

In maize roots fed ammonium, at low pH PEPCK increases from undetectable amounts to high abundance, and the amount of PEPC activity is approximately doubled. In contrast, there is no induction of the malic enzymes, and cytosolic NADP–malic enzyme decreases (Walker *et al*., 2001). This suggests that PEPCK is more important than the malic enzymes in the dissimilation of malate under these conditions. In maize roots grown in hydroponic culture, PEPCK is localised in the pericycle – a tissue that lies between the outer cortical region of the root and the vascular bundles, and PEPC is abundant in the cortex (Walker *et al*., 2001). A proportion of ammonium supplied to maize roots is likely to move through the apoplast, the part of the root outside the plasma membrane of its cells, until it reaches the endodermis, a layer of cells which separates the pericycle from the cortex (Zimmermann *et al*., 2000). The endodermis possesses a Casparian strip, a thickened cell wall, which contains the hydrophobic compound suberin that may lower its permeability to water and solutes. Ammonium on reaching the Casparian strip is likely to have to enter the cytosol. The assimilation of ammonium into glutamate in the pericycle would lead to two protons being produced, if malate and pyruvate, required for α-ketoglutarate synthesis, were synthesised from glucose. Alpha-ketoglutarate is required by the GS/GOGAT cycle, which assimilates ammonium. However, the compartmentation of PEPCK in the pericycle and PEPC in the cortex, together with the fact that similar amounts of activity of these enzymes are present, raises the possibility of transport of malate from cortex to pericyle, somewhat similar to that which occurs in a C4 leaf, in which malate is synthesised in mesophyll cells and then transported to bundle sheath cells in which it is dissimilated. The abundance of PEPCK activity in maize roots is about 0.5 μmol min^{-1} g (fresh $wt)^{-1}$; however, the enzyme is located in the pericycle, which is about 10% of the weight of the root (Walker *et al*., 2001). In these cells the activity is therefore

about 5 μmol min^{-1} g (fresh $wt)^{-1}$, which would be similar to that in cotyledons of germinating seeds, the bundle sheath of C4 plants, leaves of CAM plants, and liver and kidney in which gluconeogenesis is occurring. The import of malate into the pericycle would prevent an excess of proton production resulting from ammonium assimilation. This is because synthesis of α-ketoglutarate from imported malate or pyruvate derived from it results in ammonium assimilation no longer being a proton-producing process (see section IX.F.4). This raises the question as to why there is a need to use PEPCK, because imported malate could be used directly by the Krebs cycle, and this would make ammonium assimilation neutral with respect to proton production. The answer to this is possibly that the use of PEPCK offers greater control in preventing pH perturbations. If PEPCK was not used, the use of imported malate directly by the Krebs cycle would only be sufficient to just prevent imbalances in proton production. There would be no headroom in the mechanism, and existing excess protons could not be consumed. However, protons are still produced in the cortex by both the synthesis of malate for export and in the assimilation of ammonium. Overall the transport of malate to the pericycle would have the effect of transferring protons from the pericycle to the cortex. This may be important because of the Casparian strip at the endodermis; this acts as a barrier to diffusion between the apoplast of the pericycle and that of the cortex. This could prevent diffusion of protons – excreted into the apoplast by the cells of the pericycle – to the soil. In contrast, diffusion of protons from the apoplast of cortical cells to the soil would not encounter this problem. In roots of all the flowering plant species investigated, PEPCK was induced by feeding ammonium at pH 5.0; however, the amount induced was dependent on the species (Walker *et al.*, 2001). The anatomy of plant roots differs greatly between species and with growing conditions. For example, in maize grown in soil, an additional Casparian strip may be present at the exodermis, a layer of cells below the epidermis. These Casparian strips are thought to limit water flux through the root and consequently water loss from the leaf, and if plants are grown under a more restricted water supply then more Casparian strips may be present (Zimmermann *et al.*, 2000). This raises the possibility that although the mechanism may be metabolically similar in roots of different species or roots grown under different conditions, it could actually be compartmentalised in different cells.

Many species of flowering plant die if grown with ammonium as their only source of nitrogen. This may be because they require nitrate to counteract problems associated with excess proton production arising from ammonium assimilation. Nitrate assimilation consumes protons (Raven and Smith, 1976). Maize and rice are plants adapted to growing using ammonium as a nitrogen source. In roots of rice a Casparian strip is present at the exodermis – the layer of cells below the epidermis of the root. NADH GOGAT, an enzyme important in the incorporation of ammonium into amino acids, is localised in these cells (Ishiyama *et al.*, 1998). It is possible that, in rice, prevention of acidification problems resulting from the assimilation of ammonium in the inner tissues of the

root is alleviated by limiting the entry of ammonium into the root. In rice grown at high ammonium concentration NADH GOGAT is induced in the epidermis and exodermis, whereas GS is distributed throughout the root. This led to the suggestion that under these conditions glutamate and not glutamine is preferentially transferred to the cortex (Tobin and Yamaya, 2001).

This suggests that an additional or alternative mechanism might be important. In phloem it is thought that glutamate is metabolised by GDH and GS to form glutamine (Lohaus *et al.*, 1994). The sum of these reactions is

$$2\text{glutamate}^- + \tfrac{1}{2}O_2 \leftrightarrow \text{glutamine} + \alpha\text{-ketoglutarate}^{2-} + H_2O$$

If α-ketoglutarate is converted to OAA by the Krebs cycle and then utilised by PEPCK and the PEP so produced used in gluconeogenesis or converted to pyruvate and used by the Krebs cycle, then two protons are consumed. Glutamate would be synthesised in cells outside the Casparian strip and protons so produced excreted into the soil. The metabolism of glutamate within the pericycle would then consume protons. It is possible that both import of malate and metabolism of glutamate play a role in pH homeostasis within the pericycle. Immunolocalisation of further relevent enzymes within the maize root might assist in understanding the relative importance of the metabolism of imported malate and glutamate.

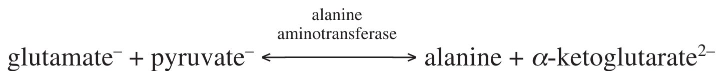
6. *The Mechanism of Proton Consumption in Roots and Kidney is Similar*

In the kidney of many mammals, PEPCK is also important in the consumption of protons by metabolic means (Silbernagl and Scheller, 1986). During acidosis protons are excreted in the urine, and these protons need to be buffered. This is achieved in proximal tubule cells by the action of glutaminase, which releases NH_4^+ from glutamine, and glutamate dehydrogenase, which releases NH_4^+ from glutamate. The NH_3 then diffuses into the urine and buffers the protons. Protons arising from NH_4^+ remain in the proximal tubule cells (Ganong, 1977; Tannen, 1978). These protons need to be removed and the way that this is achieved depends on the species and its physiological state (Silbernagl and Scheller, 1986). In some instances α-ketoglutarate arising from deamination reactions is used by the Krebs cycle, and some of the OAA produced in this way is converted to pyruvate by the action of PEPCK and pyruvate kinase. This is then returned to the Krebs cycle. Overall the use of α-ketoglutarate by the Krebs cycle in this way consumes protons. In other species and under other physiological conditions, α-ketoglutarate is used in gluconeogenesis, which requires PEPCK (Hanson and Patel, 1994). Gluconeogenesis is a proton-consuming process (Stryer, 1981). This is an important point, and raises the possibility that gluconeogenesis or potentially synthesis of lipids (from organic acids or carbon skeletons of amino acids) could serve in preventing decreases in intracellular pH in some plant tissues, if there is an excess of imported salts of organic acids or carbon skeletons of amino acids over that required for other purposes. The potential of gluconeogenesis in consuming protons is clearly illustrated in CAM

plants, in which decarboxylation of malate or citrate gives rise to CO_2 and carbon skeletons. The synthesis of malate from glucose produces two protons, and its conversion by gluconeogenesis, which utilises PEPCK, to, for example glucose, consumes two protons. Similarly, the synthesis of citrate from glucose produces three protons, and its conversion by the Krebs cycle to malate and subsequent gluconeogenesis consumes three protons. Similar considerations apply to organic acid synthesis and decarboxylation in fruit.

7. The Metabolism of Imported Amino Acids is Often Not a Proton-Neutral Process

Flowering plants transport a wide range of amino acids, amides and ureides, although in most cases the reason for this is unknown. Which nitrogenous compounds are transported depends on a number of factors, such as the species of plant, its stage of development and its physiological condition (Peoples and Gifford, 1997). However, the import and subsequent metabolism of different amino acids, amides and ureides into a tissue may produce pH perturbations. For example, the import of asparagine and its subsequent conversion to two glutamate molecules effectively transports two protons into the tissue. The import of alanine into a tissue and its conversion to glutamate or aspartate effectively transports one proton into the tissue. The transport of glutamate or aspartate into a tissue and its conversion to alanine removes one proton from the tissue. Similarly, the import of glutamine into a tissue and its conversion to two glutamate by the GS/GOGAT cycle produces two protons. It is beyond the scope of this article to perform a detailed analysis of the yield of protons for each potential interconversion. However, one example is given, and it is left to the interested reader to consider other interconversions. As an example we will consider the conversion of glutamate to alanine.



$$\text{glutamate}^- + \text{pyruvate}^- \xleftrightarrow{\text{alanine aminotransferase}} \text{alanine} + \alpha\text{-ketoglutarate}^{2-}$$

Situation 1, regeneration of pyruvate from α-ketoglutarate:

1. conversion of α-ketoglutarate to malate is proton-neutral,
2. conversion of malate to pyruvate consumes one proton,
3. overall proton yield: consumption of one proton.

Situation 2, the use of α-ketoglutarate for assimilation of ammonium arising from deamination reactions by GS/GOGAT and provision of pyruvate for alanine aminotransferase from glycolysis:

1. provision of α-ketoglutarate for assimilation of ammonium consumes two protons which would otherwise be produced in its synthesis from glucose,
2. provision of pyruvate for alanine aminotransferase from glucose produces one proton,
3. overall proton yield: consumption of one proton.

Situation 3, the use of α-ketoglutarate for assimilation of ammonium arising from deamination reactions by GS/GOGAT and provision of pyruvate from imported malate by PEPCK:

1. provision of α-ketoglutarate for assimilation of ammonium consumes two protons which would otherwise be produced in its synthesis from glucose,
2. provision of pyruvate from imported malate consumes one proton,
3. overall proton yield: consumption of three protons.

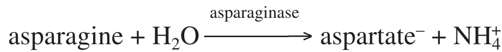
It should be noted that in situation 3 the import of malate effectively removes two protons from the tissue. This example also shows the flexibility and power of the conversion of imported malate to pyruvate by PEPCK in regulating acid base balance.

It is well established that the transport of malate between different parts of the plant is critical in the regulation of acid–base balance (Raven and Smith, 1976, and references therein). Such transport of metabolites between tissues is also important in acid–base balance in vertebrates. For example, in the Cori cycle, lactate is produced in white muscle during exercise. This produces two protons per glucose utilised; subsequently gluconeogenesis in the liver, which utilises PEPCK, consumes two protons per glucose synthesised. However, the transport of nitrogen as different amino acids, amides or ureides between plant tissues is also likely to be important in the acid–base balance of the plant. The importance of these groups of compounds in some situations is illustrated by the example of their concentration in the phloem of illuminated barley and spinach leaves. The concentration of malate is 10 mM, whereas the concentration of amino acids in barley is 186 mM and 192 mM in spinach (Lohaus *et al*., 1995). Similarly, the content of organic acids is much less than that of amino acids and amides in the phloem and xylem of *Ricinus communis* (Van Beusichem *et al*., 1988).

Developing seeds

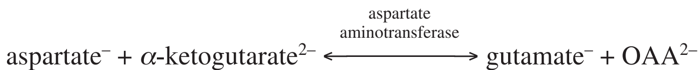
One way of looking at the pericycle of maize roots is that it is a sink for nitrogen. This then raises the question as to whether PEPCK serves a similar function in other tissues that are sinks for nitrogen (such as developing seeds). In developing seeds, imported amino acids, amides and ureides undergo extensive interconversion during their transport through the fruit and seed coat, and in both the developing embryo and storage tissues (Peoples *et al*., 1985). The reasons for such interconversions are not well understood. However, it is essential that overall they do not produce large perturbations in pH. In grape seed PEPCK is particularly abundant at the stage of development at which storage reserves are deposited, and is localised in certain cell types (Walker *et al*., 1999). In these cells the abundance is similar to the pericycle of maize root. A possibility is that in these tissues one function of PEPCK is in the prevention of perturbations in pH. The potential importance of PEPCK in the metabolism of imported assimilates was investigated further by feeding *in vitro* cultures of developing grape

seeds different forms of nitrogen. Asparagine, unlike aspartate or glutamate, gave rise to a large induction of PEPCK (Walker *et al.*, 1999). This can be explained by an examination of proton production associated with the metabolism of these compounds. Metabolism of asparagine, unlike glutamate or aspartate, potentially produces protons because NH_4^+ released by the action of asparaginase is reassimilated.



$$\text{asparagine} + H_2O \xrightarrow{\text{asparaginase}} \text{aspartate}^- + NH_4^+$$

The NH_4^+ is reassimilated by GS/GOGAT. This requires the provision of α-ketoglutarate, which, if derived from glucose, produces two protons for each NH_4^+ reassimilated. To synthesise other amino acids aspartate is likely to indergo transamination to produce glutamate.



$$\text{aspartate}^- + \alpha\text{-ketogutarate}^{2-} \xleftrightarrow{\text{aspartate aminotransferase}} \text{gutamate}^- + \text{OAA}^{2-}$$

α-Ketoglutarate may be synthesised from glucose, which produces protons, or from OAA using PEPCK and pyruvate kinase to form pyruvate for the citrate synthase reaction of the Krebs cycle, which would consume protons. It is likely that α-ketoglutarate is synthesised by both routes and the fluxes through each are balanced to produce the optimum pH within the seed achievable under these conditions. Similarly, PEPCK is abundant in the fruit and the enclosed seed of many other plant species. In pea, in which PEPCK is present in the vasculature of the pod and in the seed, a large proportion of imported nitrogen is in the form of asparagine, and PEPCK could function in a similar way.

Transport tissues

PEPCK is sometimes enriched in the vascular transport system of plants, and this depends on the part of the plant, the species and its physiological state. PEPCK is usually much more abundant in vascular tissue that is delivering assimilates to developing sinks, such as the stalk and vasculature of grape berries and the pedicel of maize kernel (Walker *et al.*, 2001). These observations are consistent with the proposed function in the metabolism of developing seeds. In flowering plants the xylem and phloem is usually contained in vascular bundles; however, an unusual exception is the curcurbit family (for example, cucumber, squash and melon). In these there are, in addition to phloem contained in the vascular bundles, phloem elements called the extrafascicular phloem, which are not contained in vascular bundles (Chen *et al.*, 2000). In the companion cells of this phloem from all parts of the plant, PEPCK is very abundant (Ehleringer and Monson, 1993). The proposal that genes encoding enzymes involved in C4 photosynthesis possess the necessary regulatory elements in C3 plants to direct their expression in C4 plants, and that this facilitated the evolution of C4 photosynthesis (Hibberd and Quick, 2002), seems somewhat at variance with the wealth of data that does not support this view (see, for example,

Westhoff *et al*., 1997). In these mechanisms PEPC is often responsible for synthesis of organic acids, although it is possible that there are exceptions. The extrafascicular phloem consists of cells termed ‘sieve elements’ and their associated companion cells. In the companion cells from petioles, stems and roots, PEPCK is at high abundance. In these cells the abundance was similar to that in the pericycle of maize root. This may be related to its anatomy; in phloem contained in vascular bundles, excess protons produced by metabolism can be transported into the apoplast and effectively removed by the xylem and consumed by metabolic processes, such as nitrate assimilation occurring elsewhere in the plant. Because the extrafascicular phloem is isolated, such exported protons are not so easily removed and the phloem may be more dependent on its own metabolism to remove protons. This could be achieved by the conversion of some of the malate or carbon skeletons of the amino acids that it transports, to PEP or pyruvate, utilising PEPCK. This would consume protons as described above.

8. Defence Tissues

In defence tissues, PEPCK may be involved in the regulation of intracellular pH, but the mechanism by which this is achieved is possibly different. PEPCK is often very abundant in defence tissues such as the trichomes of both cucumber and tobacco (Chen *et al*., 2000). PEPCK is present in trichomes from some plant species but not others, or even only in certain types of trichomes from the same species. The reason for this may be that different trichomes synthesise different secondary metabolites. An important factor that assists in the study of the function of PEPCK in trichomes is that by just scraping them off a leaf, highly purified preparations of a specific cell type can be obtained (Chen *et al*., 2000). Large amounts of secondary metabolites are synthesised in many trichomes. The synthesis of secondary metabolites transiently overproduces protons, which in many cases leads to a decrease in intracellular pH (Sakano, 2001, and references therein). This is thought to be a result of the transient overproduction of (NAD(P)H + H^+) that occurs during the synthesis of secondary metabolites (Sakano, 2001). The reason for this is that the synthesis of carbon skeletons of these compounds produces large amounts of NAD(P)H, which is far more than is required for their synthesis (Sakano, 2001). In trichomes dissected from cucumber leaves, PEPC is at low abundance, whereas PEPCK is very abundant (Chen *et al*., 2000; R. P. Walker, unpublished observations). This suggests that in these trichomes the anaplerotic replenishment of the Krebs cycle is not fulfilled by the synthesis of OAA by PEPC. A factor to consider is that if the NADH/NAD ratio in these cells is high (Sakano *et al*., 2001), then this would suggest that the OAA concentration is low (this is because of the equilibrium position of the malate dehydrogenase reaction [malate] × [NAD] = constant × [OAA] × [NADH]). For PEPCK to act as a decarboxylase would probably require aspartate to be imported and the cells to be enriched in aspartate aminotransferase, so that the concentration of OAA in the cytosol would not be too

low. If this were the case, PEPCK could then provide PEP, which could either be used by the Shikimate pathway or the Krebs cycle, and so consume protons. A problem with this is that the synthesis of secondary metabolites is proposed to only transiently overproduce protons, whereas this method permanently removes them. This leads us to consider what advantage PEPCK would have over PEPC, in this situation, if it acted as a carboxylase. It seems unlikely that it is because PEPCK produces ATP, because large amounts of NADH are produced in these cells, and there should be no shortage of oxygen. If OAA is produced by PEPCK then it will be converted to malate by malate dehydrogenase. This consumes (NADH + H^+) and will therefore increase cytosolic pH. The action of both PEPCK and PEPC would have this effect. However, if the cytosolic NADH/NAD ratio decreases then the cytosolic concentration of OAA will increase, which will enhance the decarboxylase activity. The cytosolic NADH/NAD ratio could, therefore, determine the direction of flux through the PEPCK reaction. In this way PEPCK could set the pH of the cytosol and and its redox state. However, the source of PEP for PEPCK needs to be considered. If it arises from glycolysis this, on first examination, might be thought to produce as much (NADH + H^+) as malate dehydrogenase would consume. However, this would probably not happen because a high concentration of (NADH + H^+) would cause glyceraldehyde-3-phosphate dehydrogenase to act in the direction of gluconeogenesis (Dennis *et al*., 1997). Therefore, a high NADH/NAD ratio would cause consumption of PEP by both gluconeogenesis and PEPCK. A low NADH/NAD ratio would have the opposite effect. The system therefore oscillates. This concept of oscillations is not new, and oscillations of the NADH/NAD ratio have been shown to occur in glycolysis in animals (Newsholme and Start, 1973 and references therein).

9. Concluding Remarks on the Metabolic Regulation of pH

The presence of PEPCK in acidotic tissue is likely to be dependent on the tissue and the cause of the pH perturbation. In roots subjected to anaerobic conditions, cytosolic pH falls and ATP is synthesised by substrate-level phosphorylation (Dennis *et al*., 1997). However, PEPCK is not induced in maize roots (Walker *et al*., 2001) or pea roots by anaerobiosis (Smith, 1985). In roots subjected to anaerobiosis, in contrast to those assimilating ammonium, biosynthetic reactions are curtailed (Smith and ap Rees, 1979) and the main requirement of the mechanism used to counteract acidification is to consume protons and to regenerate ATP. This shows that in maize roots a fall in cytosolic pH is not the only factor involved in the induction of PEPCK. In tissues in which PEPCK is used to decarboxylate OAA, OAA could arise from imported malate, or from the interconversions of imported amino acids. PEPCK and associated metabolism could consume protons in a number of ways, which are dependent on other metabolic processes that are occurring in the tissue and the organism as a whole.

A difficult question is why PEPCK and not the malic enzymes are used in the decarboxylation of organic acids in many situations. The answer to this is uncertain; however, there are a number of possibilities. Firstly, PEPCK produces PEP and malic enzyme produces pyruvate; this may be important in certain situations. Secondly, the compartment in which protons are consumed may be important in terms of intracellular pH balance (Netting, 2002). Thirdly, whether NADH or NADPH is formed and in which compartment may be important. Flowering plants contain plastidic and cytosolic NADP–malic enzymes and a mitochondrial NAD–malic enzyme. The importance of these factors is likely to depend on the tissue and metabolic situation. In this context, the compartment in which NADH is produced is thought to be the reason why in liver there are cytosolic and mitochondrial forms of PEPCK (Hanson and Patel, 1994).

During the evolution of flowering plants, the synthesis of organic or amino acids, their transport within or between cells and their subsequent dissimilation have been important building blocks of a number of mechanisms. Examples are C4 photosynthesis, CAM, and the metabolic regulation of pH. The ease by which these mechanisms evolve is illustrated by the fact that C4 photosynthesis and CAM have evolved independently many times in flowering plants (Ehleringer and Monson, 1993). In these mechanisms PEPC is often responsible for synthesis, although there may be exceptions. Organic acids are dissimilated by either the malic enzymes or PEPCK. The reason why PEPCK or the malic enzymes are used for dissimilation is not known for any of these mechanisms. During the colonisation of the land by plants, there was a need to develop mechanisms that were capable of transferring protons between tissues, in order to prevent pH imbalance. This was because protons could no longer be transferred directly to the surroundings as they could in the aquatic Charophycean algae, which are thought to be the ancestors of flowering plants (Raven *et al.*, 1992). The mechanism by which protons are effectively transported between different parts of the plant is likely to involve the transport of organic acids, amino acids, amides and ureides. In sink tissues, PEPCK is often induced by acidosis, and is likely to be involved in the interconversion of these imported assimilates in ways that lead to the consumption of protons. Interestingly, PEPCK functions in the regulation of acid–base balance in animals in mechanisms that are metabolically similar. A consideration of how metabolic processes interact to achieve neutrality with respect to the production of protons has received little attention and is generally poorly understood. A consideration of how overall neutrality is achieved may in some cases help us to understand the function of an enzyme, the metabolism of a tissue and the reason why metabolism is arranged in the way that it is.

10. Concluding Remarks on the Physiological Role of PEPCK in Flowering Plants

An interesting fact is that in determining the function of PEPCK in a certain tissue or type of cell it is often observed that PEPCK functions in more than

one process. For example, in leaves of CAM plants, PEPCK provides CO_2 for the Benson–Calvin cycle, and the PEP that it produces is utilised by gluconeogenesis. The function of CAM is to provide CO_2 for photosynthesis; gluconeogenesis merely facilitates this. Similarly, it is known that gluconeogenesis occurs in certain developing seeds, such as grape. However, this observation in itself tells us little. What is important is why gluconeogenesis occurs and what overall mechanism gluconeogenesis is a component of. In tissues in which large amounts of amino acids are imported, such as developing seeds, conversion of OAA derived from their carbon skeletons to PEP, by PEPCK, and conversion of PEP to sugars or pyruvate, does occur. However, its primary function may be regulation of pH associated with amino acid interconversions rather than gluconeogenesis which is just a component of the overall mechanism. It will be interesting to see how studies with mutant or transgenic plants, containing altered amounts of PEPCK, contribute to our real understanding of the physiological role of PEPCK in flowering plants. In mutant plants which contain no PEPCK (if they do not utilise the C4 and CAM photosynthetic pathways), the following effects can be predicted from our existing knowledge of PEPCK. In germination it is likely that a plant lacking PEPCK just respires OAA produced by fat and protein breakdown. This was shown using the inhibitor of PEPCK, 3-mercaptopicolinic acid (Trevanion *et al.*, 1995b). If the seed contains sufficient carbohydrate reserves then it is likely to germinate and grow normally; if it does not it is likely to do so if fed sugar. In the mature plant, a lack of PEPCK may lead to flower or seed abortion. These symptoms are likely to be more severe in plants grown on ammonium rather than nitrate. Root growth is also likely be impaired on plants grown on ammonium. In transgenic plants, it is predicted that there will be little effect until PEPCK is reduced by more than 90%, because flux through the enzyme will be increased by decreased phosphorylation and an increase in OAA concentration. It has been found that a number of flowering plants contain more than one gene for PEPCK, and it has already been shown that in one species these genes are expressed in different tissues (Finnegan *et al.*, 1999). It seems likely that studies with other plant species will tell a similar story. What is now more challenging is to determine whether these different forms of PEPCK have properties that suit them to a certain metabolic role. From our present knowledge of PEPCK it would seem likely that forms of PEPCK that are not subject to phosphorylation will be expressed in tissues in which regulation by a substrate cycle between the carboxylase and decarboxylase activities of PEPCK is likely to be important, such as the leaves of C4 plants.

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Developmental Genetics of the Angiosperm Leaf

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ABSTRACT

The leaf is the major site of photosynthesis and the prototypical organ of terrestrial plants. The past five years have seen a dramatic increase in our understanding of the genetic control of leaf development. The establishment of determinacy, the role of the meristem, the establishment of polarity, the control of cell division, vascularization and epidermal

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patterning have all been the subject of genetic screens and many mutants have been isolated and characterized. Many of these mutants affect floral and embryonic structures, demonstrating the inherent similarities in all lateral organs. In this review we focus on the interactions between these genes and the networks that establish pattern in lateral organs.

I. INTRODUCTION

Most of the sunlight that is captured by plants is absorbed by algae in the oceans, but on land sunlight is captured by leaves, and the arrangement of photosynthetic cell types is a predominant factor in the efficiency of this process. The first terrestrial plants appeared about 440–410 million years ago, during the early Devonian. The dry, hostile environment of land required several adaptations. In contrast to seaweeds and freshwater algae, land plants developed a cuticle to provide a waterproof coating. The epidermis was punctuated with stomata to allow gas and water exchange. A strong vascular system supplied them with water and minerals from the soil as well as mechanical support. However, the plants of the early Devonian differed from most modern plants in that they lacked leaves (Gifford and Foster 1989; Bateman *et al.*, 1998).

The evolution of leaves coincided with a fall in CO_2 levels that occurred during the late Paleozoic. Early Devonian land plants had few stomata and no leaves, while the planate leaves of the late Devonian had much higher densities of stomata. Beerling *et al.* (2001) modeled the gas exchange and heat absorbance of fossil plants, based on their size, vascular systems and stomatal densities. The low density and inefficient vascular system of early Devonian plants would not have been able to cool leaves by transpiration. Lower levels of CO_2 require more stomata to absorb enough gas for efficient photosynthesis, and in modern plants low CO_2 levels cause more stomata to develop (Gray *et al.*, 2000). Thus the Paleozoic drop in CO_2 levels would have favoured evolution of a higher density of stomata. Increased cooling efficiency would allow the evolution of laminate leaves. Consistent with this idea the earliest fossil laminate leaves are found in cool northern latitudes and are finely divided (Hao and Beck, 1993).

Theories of plant morphology divide the terrestrial plant body into three parts. The rhizome is the indeterminate product of the root pole, the caulome is the indeterminate product of the shoot pole and the phyllome is a determinate organ produced by the shoot (Goethe, 1764 cited in Arber, 1950; Sattler 1988). Although this classification is probably too rigid to encompass all plant morphology (Bell, 1991), developmental and genetic studies have supported the homology of all lateral organs of the plant (Sattler, 1988; Honma and Goto, 2001; Pelaz *et al.*, 2001). The early development of sepals, petals, carpels and many thorns and tendrils is similar to that of leaves. Furthermore, genes that are required for early steps in leaf development (initiation, outgrowth and patterning) are also required for the development of other lateral organs. In some cases

genes are redundant with additional family members in the leaf, but act alone in the floral organs. Mutations in these genes have a floral but not a vegetative phenotype. This situation may reflect the derivation of flowers from leaves. Information gained from studies of floral development can therefore be used to understand leaf development, while leaf development has relevance for flowers and seeds.

Early studies on leaf development were largely descriptive (reviewed in Arber, 1950; Kaplan, 2001). Later, micro-dissection, irradiation, radiolabeling and organ culture were used to study a variety of vascular and non-vascular plants. These studies helped to define some of the central concepts in leaf development (Steeves and Sussex, 1989; Maksymowych, 1973). For example, signaling between the meristem and the developing primordium was found to be important for axis specification (Sussex, 1954), while cell division was not (Foard, 1971). Lateral organs were found to be determined gradually (Sachs, 1969) and auxin was found to play a prominent role in vascular specification (Sachs, 1975). However, research in leaf development has advanced considerably with the adoption of model genetic systems. Maize, *Antirrhinum*, pea, tomato, rice and *Arabidopsis* have all been used to study the genetics of leaf development, and the past five years have seen a mechanistic model begin to emerge. In this review we describe advances in understanding the genetic basis of the major processes of leaf development; the acquisition of a determinate fate, the establishment of polarity, and growth and differentiation.

II. COMPARATIVE ANATOMY OF THE MATURE LEAF

Leaves have three major axes along which morphological differentiation occurs. The proximodistal axis lies along the length of the leaf and is the primary axis of growth. The mediolateral axis typically exhibits bilateral symmetry and can also permit extensive growth. By contrast, the dorsoventral axis, which extends across the leaf from the abaxial to the adaxial surface, is much shorter, consisting of only a handful of distinct cell layers (Fig. 1). Leaves vary in the degree of morphological specialization along each axis.

Some leaves vary little along the proximodistal axis, for example the strap-like leaf of the spider plant *Chlorophytum* clasps the stem at the proximal end and tapers gradually to a point at the distal end. Leaves of the tropical pitcher plant *Nepenthes spp.* are at the opposite extreme, comprising alternating flattened and radial zones to form a complete pitcher (Owen and Lennon, 1999). Most plants fall somewhere between these two extremes. Some dicots have a proximal sheath, which strengthens the connection to the stem. Distal to the sheath is a petiole, which allows free movement of the leaf. Distal-most is an expanded lamina which can show variation in shape; for example, from a broad base to a pointed tip for guttation. A pair of stipules is usually found at the proximal end of the petiole on the adaxial side of the leaf (Bell, 1991) (Plate 1).

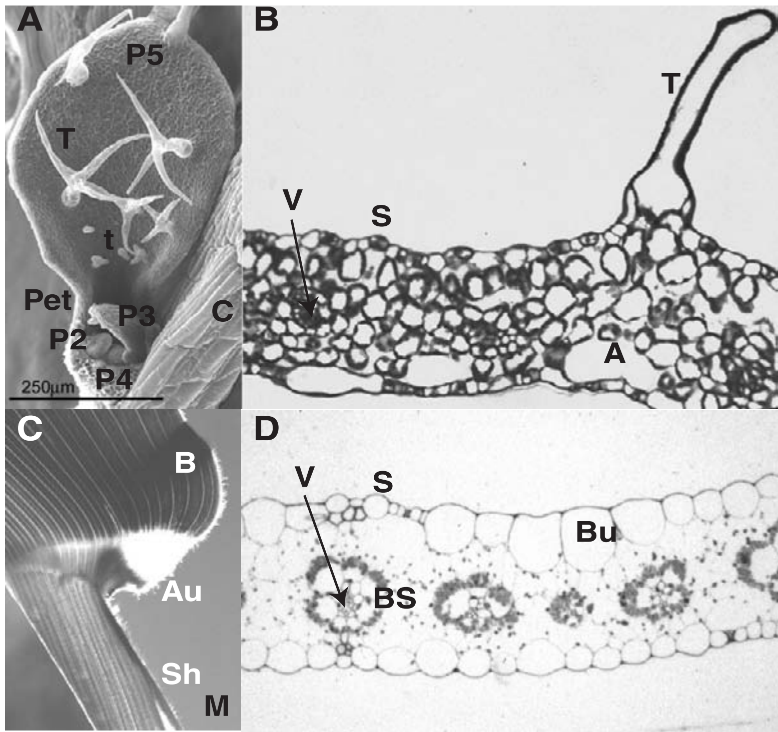

Fig. 1. Patterning along the developmental axes of the leaf. A. SEM of a seven-day-old *Arabidopsis* seedling, showing the arrangement of leaf primordia around the shoot apical meristem (SAM) (leaf P4 has been removed). The petiole (Pet) is distinct from the blade in the older leaf (plastochron (P5). A proximodistal gradient can be seen, from young trichomes (t) at the proximal end of the leaf to mature trichomes (T) at the distal end. The petiole of the cotyledon (C) runs to the right. B. Transverse section through the first leaf from a 7-day-old *Arabidopsis* seedling. Stomata (S) develop on both the adaxial and abaxial surfaces. A trichome (T) can be seen on the adaxial surface. Air spaces (A) are present between spongy mesophyll cells on the abaxial side of the leaf, whereas palisade cells on the adaxial side are densely packed. Veins (V) form in the middle of the leaf. C. The junction between blade and sheath in a maize leaf. The hinge between the expanded blade (B) and the narrow, rolled sheath (S) is the auricle (Au). Also the parallel venation is apparent. D. Transverse section through a young maize leaf. Stomata (S) can be seen on the adaxial surface. Although not visible in this section they occur equally abundantly on the abaxial surface. Bulliform cells (Bu) are produced on the adaxial surface. The veins (V) are surrounded by concentric rings of bundle sheath cells (BS), which contain large, centrifugally arranged chloroplasts.

In many plants stipules protect the developing bud, but they can also be modified to fulfil other roles such as leaflets (pea), nectaries (*Bauhina* spp.), or ant homes (*Acacia* spp.).

Leaves of grass species such as maize and rice are divided into a proximal region – the sheath – and a distal region – the blade. The sheath wraps around the stem, providing support for the blade which projects outwards to catch the light. Blade and sheath are separated by the auricle and the ligule. The auricle is a hinge that allows the blade to bend outwards. The ligule is a hairy fringe on the adaxial side of the leaf that protects the axillary bud, wrapped within the sheath, from predators and falling particles.

The basic form of leaves is either simple, where the blade or lamina is not divided, or compound, where the lamina is divided into leaflets. It has been proposed that the ancestral state for angiosperms is a simple leaf and that compound leaves arose multiple independent times in angiosperm lineages (Hofer and Ellis, 1998; Bharathan and Sinha, 2001). The basic types of compound leaf are pinnate and palmate. Unipinnate compound leaves have leaflets arranged along the main axis or rachis of the leaf. Varying degrees of repetition of this pattern form higher-order compounding. In palmate leaves, the leaflets emerge from one point at the distal end of a petiole. Tomato and pea reflect different compound leaf growth patterns. The tomato leaf is characterized by three to four lateral leaflet pairs and a terminal leaflet, that develop basipetally. In contrast the pea leaf is composed of a pair of basal stipules, several pairs of lateral leaflets, two or more pairs of lateral tendrils and a terminal tendril, and development proceeds acropetally.

The contribution of different parts of the leaf primordium to the mature leaf varies from species to species. Arber (1925) first suggested that the monocot blade was homologous to the dicot petiole, but analysis of a range of species led to the formulation of the 'leaf base' model (Troll, 1939; Kaplan, 1973). This states that the leaf primordia can be divided into upper and lower leaf zones. In dicots the upper zone gives rise to the blade, the lower to the sheath and stipules. The petiole later separates these two regions. In many monocots the lower zone gives rise to the blade and the sheath, while the upper zone is substantially reduced and forms only a small unifacial structure termed the 'vorläuferspitzer' (Bell, 1991) (Plate 2). However, a study of a range of monocot leaves shows that in some species it is the upper leaf zone which forms the lamina (Bharathan, 1996).

Most leaves are dorsoventrally flattened to maximize the capture of sunlight and exchange of gas. Typically, leaves have several layers of ground tissue between the adaxial (top) and abaxial (bottom) epidermis. The two epidermal surfaces can differ greatly; for instance, marram grass has hairs and stomata only on the abaxial side (Bell, 1991). Internally, many dicots have tightly packed palisade cells on the sunlit adaxial side of the mesophyll, and less-dense spongy mesophyll cells for gas exchange on the shaded abaxial side. Polarity within the vascular bundles results in adaxial xylem and abaxial phloem.

Given the huge diversity of leaf anatomy, are the underlying mechanisms of pattern formation conserved? Recent studies have revealed that these mechanisms operate in the very early primordia, as well as in the shoot meristem, and they are conserved to at least some degree even between monocots and dicots. These mechanisms are described in detail below.

III. LEAF DETERMINATION AND INITIATION

Shoots are characterized by indeterminate growth, whereas leaves typically develop as determinate organs. Only a few extant plants, such as members of the family Tristichaceae (Rutihauser and Huber, 1991) and the genus *Welwitschia* (Rodin, 1958), have indeterminate leaves or leaf-like organs. The reason for a major evolutionary trend toward determinate organs is not clear. One contributing factor is that indeterminate leaves, with exposed growing tips, may be more vulnerable to mechanical damage and predation. This problem would be circumvented by development of a determinate organ, with cell divisions confined to the base. Thus the evolution of leaves involved a change from the dichotomizing tip growth typical of early land plants (Bold and Wynne, 1985; Bateman, 1998) to the formation of leaves in the context of a shoot meristem.

A. THE SHOOT APICAL MERISTEM

The shoot apical meristem (SAM) comprises undifferentiated and slowly dividing stem cells at the tip that give rise to peripheral derivatives from which founder cells for organ formation are recruited. The SAM is organized into central and peripheral zones, distinguished by cell division rates and gene expression patterns (reviewed in Bowman and Eshed, 2000; Clark, 2001). Superimposed on this organization are discrete cell layers. The outermost L1 layer gives rise to the epidermis, while inner L2 and L3 (and in some species higher-order layers) contribute mostly mesophyll and vascular tissues, respectively. In grasses and some other species, the L1 contributes to inner tissues near the margins of the leaf (Tilney-Bassett, 1986).

The *knox* family of transcription factors are required for SAM function. These are TALE class homeobox proteins related to the MEIS1 superfamily in animals. Phylogenetic analysis has revealed two classes of *knox* genes in plants (Kerstetter *et al.*, 1994; Bharathan *et al.*, 1999; Reiser *et al.*, 2000). Only class I genes are implicated in indeterminacy as they are expressed predominantly in the SAM. Loss-of-function mutations as yet have not been identified in the class II *knox* genes, but they are expressed in a wide variety of tissues, suggesting that the role of these genes is distinct from that of class I genes.

knox genes have been identified in all plant species examined thus far. Members of the class I and class II *knox* genes exist in mosses, indicating that divergence between these two classes occurred at least 400 million years ago (Champagne and Ashton, 2001). The early evolution of *knox* genes in plants is also suggested by the identification of a family member in the unicellular alga *Acetabularia acetabulum* (Serikawa and Mandoli, 1999).

The first *knox* gene identified was *knotted1* (*kn1*) of maize (Vollbrecht *et al.*, 1991). Significantly *kn1* is expressed in the indeterminate cells of the SAM and down-regulated in determinate lateral organ primordia (Smith *et al.*, 1992;

Jackson *et al.*, 1994). Loss-of-function mutations in *kn1* reduce meristem function, implicating *kn1* in initiation and/or maintenance of the SAM (Kerstetter *et al.*, 1997; Vollbrecht *et al.*, 2000). Of the four class I *knox* genes in *Arabidopsis* (*SHOOT MERISTEMLESS* (*STM*) *KNAT1*, *KNAT2* and *KNAT6*), only *STM* has been demonstrated to be essential for SAM function (Barton and Poethig, 1993; Long *et al.*, 1996). Strong mutant alleles of *STM* do not have a SAM and fail to produce a shoot. As with *kn1* in maize, *STM* is expressed in embryo, vegetative, inflorescence and floral SAMs and down-regulated in founder cells and lateral organ primordia. *KNAT1*, *KNAT2* and *KNAT6* are also expressed in SAMs, but the pattern and timing of expression differs from that of *STM* (Lincoln *et al.*, 1994; Dockx *et al.*, 1995; Reiser *et al.*, 2000; Semiarti *et al.*, 2001). Mutations in *KNAT1* and *KNAT2* have no meristem defects; however, *KNAT1* is conditionally redundant with *STM*, and can play a role in SAM function (see section III.B).

B. FOUNDER CELLS

The first step in leaf development is the recruitment of cells from the meristem to form the incipient leaf primordium. The extent to which cells around the circumference of the meristem are recruited into the developing leaf primordium varies, and may contribute to different leaf forms. The two extremes of lateral founder cell recruitment are apparent in *Arabidopsis* and maize. In *Arabidopsis* only 12–30 founder cells contribute to the mature leaf (Furner and Pumfrey, 1992; Irish and Sussex, 1992). Leaf primordia curve over the SAM, but the base of each successive leaf does not overlap (Plate 1). In comparison, cell recruitment occurs around the entire circumference of the maize SAM, with about 250 founder cells contributing to the mature leaf (McDaniel and Poethig, 1988) (Plate 1, Plate 2). The base of each leaf completely sheaths the SAM.

Founder cells appear to establish determinacy by switching off meristem-specific genes and initiating a new program of organ-specific gene expression. For example, *knox* genes are down-regulated, while genes specific to lateral organs (such as *ANITEGUMENTA* (*ANT*), *LEAFY* (*LFY*) and *ASYMMETRIC LEAVES1* (*AS1*)) are expressed. Between these two domains of gene expression a boundary region defines an area of low cell division. This is thought to prevent organ fusion (Martienssen and Dolan, 1998; Barton, 2001) and limit founder cell lineages to organs (Vincent *et al.*, 1995).

Down-regulation of the *knox* family is a very early marker of organ formation. Genes regulating the initial down-regulation of *knox* genes are yet to be identified; however, a number of mutations in maize partially affect this process. The two redundant genes *narrow sheath1* (*ns1*) and *narrow sheath 2* (*ns2*) are required for lateral founder cell recruitment, such that double mutants form narrow leaves that lack margins (Scanlon *et al.*, 1996; Scanlon and Freeling, 1997). In these plants *kn1* is down-regulated at the site of leaf initiation, but founder cells are not recruited in the marginal domains of the leaf. Mosaic

analysis has shown that *ns1* acts in two small foci on the meristem flanks to propagate founder cell recruitment (Scanlon, 2000). The *leafbladeless1* (*lbl1*) mutant in maize also has limited founder cell recruitment and this is coincident with defects in leaf dorsoventral polarity (Timmermans *et al.*, 1998) (see section IV.B.1). As lateral cell recruitment is not as extensive in *Arabidopsis* or *Antirrhinum* leaf primordia, the effect of mutations in *ns1* and *ns2* homologs in these species is difficult to predict. Mutations in genes required for down-regulation of *knox* genes at the site of leaf initiation may fail in cotyledon development and therefore be lethal to the seedling.

In species with simple leaves, *knox* genes remain down-regulated in leaf primordia. However, in the compound leaf of tomato, *knox* gene expression persists after leaf initiation (Hareven *et al.*, 1996; Janssen *et al.*, 1998; Goliber *et al.*, 1999; Bharathan and Sinha, 2001). In contrast, *knox* genes are not expressed in the compound leaves of pea, which instead express homologs of *LFY*. *LFY* family members are required in many species for the transition from vegetative to inflorescence growth (Coen *et al.*, 1990; Weigel *et al.*, 1992; Blazquez *et al.*, 1997; Molinero-Rosales *et al.*, 1999), but mutations in *UNIFOLIATA* (*UNI*), the pea homolog of *LFY*, create a simple leaf phenotype (Hofer *et al.*, 1997; Gourlay *et al.*, 2000). *knox* genes and *UNI* therefore appear to play a role in the formation of two different types of compound leaf.

Evidence that regulation of *knox* gene expression in lateral organ primordia is critical for patterning lateral organs also comes from ectopic expression studies. In dicot species with simple leaves such as *Arabidopsis*, tobacco and lettuce misexpression of *knox* genes results in leaf lobing (Sinha *et al.*, 1993; Chuck *et al.*, 1996; Frugis *et al.*, 2001), whereas ectopic expression of *knox* genes in the compound leaf of tomato leads to an increase in the degree of compounding (Chen *et al.*, 1997). Misexpression of *knox* genes in grasses result in alterations to leaf patterning (Hake and Freeling, 1986; Hake *et al.*, 1989). In maize, dominant neomorphic mutations cause ectopic expression in leaves, leading to over-proliferation of cells and displacement of proximal cell fates distally (Freeling, 1992; Müller *et al.*, 1995; Williams-Carrier *et al.*, 1997; Sentoku *et al.*, 2000). In addition, in many cases misexpression of *knox* genes is associated with the formation of ectopic shoots on lateral organs. Misexpression of *knox* genes causes misregulation of several hormones that may contribute to the phenotypes observed (Kano-Murakami *et al.*, 1993, Ori *et al.*, 1999; Sakamoto *et al.*, 2001).

Several genes that negatively regulate *knox* genes have been identified. Mutations in *PHANTASTICA* (*PHAN*) in *Antirrhinum, rough sheath2* (*rs2*) in maize, *CLAUSA* in tomato, and *AS1* and *ASYMMETRIC LEAVES2* (*AS2*) in *Arabidopsis*, all show ectopic *knox* gene expression in the leaf (Timmermans *et al.*, 1999; Tsiantis *et al.*, 1999; Avivi *et al.*, 2000; Byrne *et al.*, 2000; Ori *et al.*, 2000; Semiarti *et al.*, 2001). *PHAN, RS2* and *AS1* encode closely related myb domain proteins. However, mutations in these genes confer different phenotypic defects. *phan* affects leaf outgrowth and dorsoventral patterning (Waites and Hudson, 1995; Waites *et al.*, 1998). In contrast, *as1* mutants show

no apparent dorsoventral defects, but their leaves are lobed (Byrne *et al.*, 2000; Ori *et al.*, 2000; Tsukaya and Uchimiya, 1997). One interpretation is that *as1* leaves have reiterated the proximodistal axis. Although *Antirrhinum* and *Arabidopsis* are both dicots, they are distantly related. Thus the disparity between *phan* and *as1* phenotypes could reflect different or divergent functions for their targets. Importantly, maize *rs2* mutants also display only proximodistal defects, with displacement of the proximal sheath, auricle and ligule tissues distally into the leaf blade (Schneeberger *et al.*, 1998; Timmermans *et al.*, 1999; Tsiantis *et al.*, 1999).

The variegated pattern of *knox* gene deregulation in *rs2* null mutants indicates that *rs2* acts as an epigenetic regulator to maintain *knox* gene silencing (Schneeberger *et al.*, 1998; Timmermans *et al.*, 1999). In support of this, the phenotype of *as1* and *as2* mutants is enhanced by mutations in two genes, *PICKLE* (*PKL*) and *SERRATE* (*SE*) (Ori *et al.*, 2000). These genes are predicted to be required for proper chromatin structure suggesting targets of *knox* gene regulator*s* are sensitive to chromatin states.

Although *PHAN, rs2* and *AS1* function as negative regulators of *knox* genes in *Arabidopsis, STM* also negatively regulates *AS1*, as well as *AS2*. Both genes suppress the meristem defect of *stm* mutants. At least in the case of *as1* suppression is dependent on *KNAT1* (Byrne *et al.*, 2000, 2002). Mutations in *KNAT1* alone have no obvious vegetative meristem phenotype (Byrne *et al.*, 2002; Douglas *et al.*, 2002; Venglat *et al.*, 2002). Therefore *KNAT1* is redundant with *STM* in the shoot, but only in the absence of *AS1*.

These interactions suggest a mechanism by which indeterminate cells in the SAM and determinate leaf founder cells are distinguished. In meristem cells *STM* negatively regulates *AS1* which, in turn, down regulates other *knox* genes. Mutually negative interactions of this sort are used to establish territorial boundaries in animals, and may establish a boundary between stem cells and founder cells in the meristem (Martienssen and Dolan, 1998; Barton, 2001; Byrne *et al.*, 2001). This boundary, as well as delineating these two domains defines an area of reduced cell division between organs and so contributes to organ separation (Vincent *et al.*, 1995). *NO APICAL MERISTEM* (*NAM*) from petunia, *FIMBRIATA* (*FIM*) from *Antirrhinum* and *UNUSUAL FLORAL ORGANS* (*UFO*) and *CUP-SHAPED COTYLDEONS* (*CUC1* and *CUC2*) from *Arabidopsis* are expressed in the boundary region throughout development. Mutations in these genes result in organ fusion (Ingram *et al.*, 1995; Levin and Meyerowitz, 1995; Souer *et al.*, 1996b; Ingram *et al.*, 1997; Aida *et al.*, 1997, 1999). Another gene expressed in this domain is *LATERAL ORGAN BOUNDARIES* (*LOB*). Mutations in this gene have no phenotypic effect, but this may be due to redundancy as *LOB* is a member of a large gene family (Shuai *et al.*, 2002). *UFO* lies downstream of *STM*, while the *CUC2* and *NAM* genes are upstream (Souer *et al.*, 1996a; Aida *et al.*, 1997; Long and Barton, 1998). As a result, mutations in *NAM* and *cuc1/cuc2* double mutants give shoot meristemless phenotypes (Souer *et al.*, 1996a; Aida *et al.*, 1997). Expression of *LOB* is positively

regulated by both *AS1* and *KNAT1* suggesting that gene expression patterns in this region depend on the interaction of meristem cells and founder cells (Byrne *et al.*, 2002; Springer and Martienssen, unpublished).

C. LEAF INITIATION AND PHYLLOTAXY

The first visible sign of leaf initiation is a change in the orientation of cell divisions in the peripheral region of the SAM. The outer layers of the meristem, which principally undergo anticlinal divisions, initiate divisions in a plane parallel to the meristem surface. Although the rate of cell division in the peripheral zone of the meristem is higher than in the central zone, the rate of cell division increases further in leaf primordia. Lateral organs initiate on the flanks of the SAM in predictable phyllotactic patterns that vary with species and developmental phase. Primordia initiate in a number of discrete patterns, distichous (single leaves alternating sides at each node), whorled (including decussate which is a whorl of two leaves at one node) and spiral. In general, phyllotactic patterns can be described in terms of opposing intersecting spirals corresponding with successive numbers in a Fibonacci series (Jean, 1994). Similar patterns are formed in other biological systems, including the pattern of branching in seaweeds and in non-living systems (Church, 1904; Thompson, 1917; Jean, 1994).

It is still not clear how the phyllotactic pattern is first established and what factors influence changes in phyllotaxy over time. However, surgical experiments have shown that leaf primordia are positioned in response to pre-existing primordia (Snow and Snow, 1931, 1933, 1935). This phenomenon can also be demonstrated genetically. Mutations in the *Arabidopsis* gene *FOREVER YOUNG* (*FEY*) cause necrosis in the SAM. Primordia are initiated abnormally, and loss of spacing between primordia results in a reversal of the phyllotactic spiral (Callos *et al.*, 1994).

Establishment and maintenance of phyllotactic patterns have been postulated to be biophysical in nature. One theory postulates that organs initiate as a result of compression stresses within the meristem (Green, 1999) These forces are reflected in reorientation of cytoskeletal microtubules (Selker *et al.*, 1992; Lloyd, 1995), but mutants that disturb this pattern do not always have disturbed phyllotaxy. *FASS* and *TONNEAU* (*TON*) alleles have cytoskeletal defects that lead to irregular cell expansion and division (Torres-Ruiz and Jurgens, 1994; Traas *et al.*, 1995). Cotyledons are often misplaced or increased in number, but the phyllotaxy of leaves and floral organs is not disturbed. This suggests that stresses acting on alignment of microtubules are not the mechanism of propagation of phyllotaxy, although they may be involved in initiation.

Another model postulates that preexisting organ primordia govern the position of new organs by production of a diffusible inhibitor. Chemically mediated pattern formation, based on diffusion of activators and inhibitors, was initially modeled by Turing (1952). In a plain field, expression of long-distance activators and short-distance inhibitors from a single focus results in a self-replicating

pattern of equally spaced foci (Meinhardt, 1984). The origin of a diffusible inhibitor is not known, but does not appear to be directly related to either the size of the meristem or the size of leaf primordia. For example, the mutant *plastochron1* (*pla1*) in rice has an enlarged meristem but normal phyllotaxy (Itoh *et al.*, 1998). In addition, mutants that alter the size of primordia, such as *ns* and *lbl1* in maize, are not associated with defects in phyllotaxy (Scanlon *et al.*, 1996; Scanlon and Freeling, 1997; Timmermans *et al.*, 1998). One gene which may be involved in determining leaf initiation sites is the maize gene *terminal ear* (*te*). Compared with wild type, where leaves are arranged in a distichous phyllotaxy, upper leaves in *te* mutants are initiated more frequently. *te* is expressed weakly throughout the apical dome and strongly in horseshoe-shaped stripes at the position of the incipient leaf and at the base of leaf primordia. It is excluded from the site where founder cell recruitment initiates. *te* may therefore regulate phyllotaxy by preventing leaf initiation within its domain of expression (Veit *et al.*, 1998).

Although the size and shape of the meristem do not necessarily predict phyllotactic pattern, changes in these parameters are often associated with it (Clark *et al.*, 1995; Itoh *et al.*, 2000; Helliwell *et al.*, 2001). *abphyl* is a maize mutant which has an enlarged meristem and forms leaves in a decussate pattern (Jackson and Hake, 1999). However, the phyllotaxy frequently reverts to distichous, coincident with a change in the size of the meristem. This stochastic change is similar to the change in phyllotaxy during the switch from vegetative to inflorescence growth in *Antirrhinum*. In both cases, a change in the size of the meristem occurs with the change in phyllotaxy.

Auxin is a strong candidate for a diffusible signal regulating primordia outgrowth in the meristem. Mutants in auxin signaling such as *PINOID* (*PID*), *PIN-FORMED1* (*PIN1*) and weak alleles of *MONOPTERIS* (*MP*) have defects in organ initiation and fail to initiate floral primordia (Bennett, 1995; Przemeck *et al.*, 1996). However, in *pin* and *pid*, floral genes can be detected in peripheral regions of the inflorescence meristem, revealing that patterning programs are initiated but fail to elaborate (Christensen *et al.*, 2000; Benjamins *et al.*, 2001).

Auxin is not only necessary but also sufficient for leaf primordia initiation. An elegant series of experiments has shown that localized auxin can induce lateral organ formation. Application or induction of auxin to the naked inflorescence meristem of *pin1* mutants or to meristems of tomato grown in the presence of an auxin inhibitor induces lateral organ formation at the point of localized auxin (Reinhardt *et al.*, 1998, 2000). Organs can be induced at any position around the circumference of the meristem, but competence to form an organ is restricted along the apical/basal axis of the SAM. Thus local concentrations of auxin in the meristem may govern phyllotaxy. This would explain the self-perpetuating nature of phyllotactic patterns, as auxin is produced by successive leaf primordia and by the leaf traces that in many plants predict where the next leaf will form.

One process regulated by auxin is activation of expansins which disrupt hydrogen bonds between cellulose fibers, thereby allowing for loosening of the

plant cell wall (Cosgrove, 1993; McQueen-Mason and Cosgrove, 1995; Cleland, 2001). As with auxin, localized expression of expansins in tobacco meristems induces the formation of new leaves (Pien *et al.*, 2001). Thus auxin may regulate leaf initiation by inducing localized cell expansion. This would be consistent with early studies demonstrating primordia initiation can still occur even in the absence of cell division (Foard, 1971).

IV. AXIS SPECIFICATION

A. THE PROXIMODISTAL AXIS

During leaf determination, the axes of the leaf are specified relative to the axis of the shoot. The proximodistal axis of the leaf extends out from the surface of the meristem. Only a few genes affecting the proximodistal axis have been described, and most regulate class I *knox* genes. Ectopic expression of *KNAT1* results in lobed leaves. Each lobe is subtended by stipules (Chuck *et al.*, 1996), indicating that it is a reiteration of the proximodistal axis (Martienssen and Dolan, 1998). Ectopic expression of *knox* genes in the grass leaf results in the transformation of distal to proximal cell types (Freeling, 1992; Sylvester, 1996).

The phenotypes of ectopic *knox* expression in maize leaves led Freeling to propose the 'maturation schedule hypothesis' (Freeling *et al.*, 1992). According to this model, founder cells are all competent to become sheath. As the leaf develops, distal regions acquire new competencies to form ligule, auricle, and subsequently, blade. Later, cells differentiate basipetally according to their competency. According to this model, ectopic *knox* gene expression delays the progression through different competency states. Support for this model has been obtained from mosaic analysis of *Liguleless3*. The onset of *Lg3* expression during primordium development affected cell fate in the blade region but not the timing of differentiation (Muehlbauer *et al.*, 1997).

An alternative model is that *knox* genes prolong cell division and therefore delay differentiation, and in this manner may be associated with proximodistal patterning (Barton, 2001). Many grass leaves continue to grow at the base long after the tip is fully expanded and differentiated, in a region once referred to as a 'basal meristem'. The sheath wraps around the internode, and cell division is prolonged to allow both to elongate in synchrony (Sharman, 1942). This phenomenon is not limited to grasses, and in many plant species cell division is lost progressively from the tip of the leaf along the proximodistal axis (Steeves and Sussex, 1989). The *knox* genes *gnarley* and *rs1* in maize, *OSH15* in rice as well as *KNAT1* in *Arabidopsis* are expressed in a region at the base of the initiating leaf, which may include both proximal regions of the lateral organ and internode (Jackson *et al.*, 1994; Schneeberger *et al.*, 1995; Sato *et al.*, 1998; Reiser *et al.*, 2000; Barton, 2001) (Plate 3). Consistent with the idea that *knox* genes prolong cell division loss of function mutations in *KNAT1* (*brevipedicellus* (*bp*)) have reduced pedicels (flower stalks) (Byrne *et al.*, 2002; Douglas *et al.*, 2002;

Venglat *et al.*, 2002). This suggests that *KNAT1* is required to maintain cell division in the proximal domain of floral primordia.

B. DORSOVENTRAL PATTERNING

The dorsoventral axis is also specified relative to the shoot. Cells that will contribute to the adaxial side of the leaf are closest to the tip of the SAM, whereas cells that will form the abaxial side of the leaf are further away (Plate 1). Incisions that separate the incipient primordium from the apex can result in radially symmetric, abaxialized leaves, indicating that contact with the meristem is required for dorsoventrality (Sussex, 1955). The *phan* mutation in *Antirrhinum* has radial, abaxial leaves and altered floral organs (Waites and Hudson, 1995). Weakly affected leaves have abaxial sectors on the adaxial surface surrounded by raised epidermal rims. This led Waites and Hudson (1995) to propose that, as in lateral organs in animals, outgrowth of blade margins is normally driven by the juxtaposition of abaxial and adaxial domains. When this boundary is lost radial organs are produced. Genetic studies have greatly extended these simple concepts in the past few years, and are summarized in the following sections.

1. Adaxial Determinants

Phabulosa1-D (*Phb1-D*) is a semi-dominant *Arabidopsis* mutation that has the 'opposite' phenotype to *phan*. Homozygotes have radial adaxialized leaves that often lack vascular tissues. (McConnell and Barton, 1998). *PHB* encodes a member of the plant-specific HD-ZipIII family – *ATHB14* (McConnell *et al.*, 2001). HD-ZIPIII proteins contain an N-terminal homeodomain and a leucine zipper followed by a START lipid-sterol binding domain. Based on this homology, these proteins have been proposed to function as receptors. The expression pattern of *PHB* is consistent with a role in communication between the leaf and the meristem (Plate 3). In the vegetative shoot, *PHB* expression extends in rays from the center of the meristem to the leaf primordia, where it is uniformly expressed. As the leaf develops, the 'ray' is lost and by plastochron 2 expression of *PHB* becomes confined to the adaxial side and to the vasculature. Semi-dominant alleles of the closely related gene *ATHB9* or *PHAVOLUTA* (*PHV*) share the same phenotype as *Phb1-D*, and all mutant alleles of both genes have mutations at one of two sites in the START domain (McConnell *et al.*, 2001). As ectopic expression of the normal transcript has no effect, the mutant proteins may be constitutively activated independent of a hypothetical ligand. *PHB-like* genes may be redundant, as loss-of-function mutants have relatively mild phenotypes, if any. For example, *REVOLUTA* (*REV*) encodes a PHB-like protein and *rev* mutants have large leaves but lack axillary meristems and show vascular defects (Zhong and Ye, 1999; Ratcliffe *et al.*, 2000; Otsuga *et al.*, 2001).

Several mutants that affect dorsoventral patterning have been identified in maize. Strong alleles of *lbl1* form radial abaxialized leaves (Timmermans *et al.*,

1998). As in *phan* mutants, weak *lbl1* mutants develop patches of abaxial cells on the adaxial side of the leaf surface. This can lead to bifurcation of the leaf or to formation of fully differentiated leaf lamina at the ad/abaxial boundaries. Specification of the blade/sheath boundary occurs independently in the two halves of bifurcated leaves and in the new ectopic lamina. This suggests that dorsoventral patterning affects the proximodistal axis. There are at least two additional loci that display the same phenotype (M. T. and M. Juarez unpublished data). Semi-dominant mutations in *Rolled* (*Rld*) have rolled-up leaves with reversed dorsoventral polarity. In wild type the ligule forms on the adaxial side of the leaf, and the adaxial epidermis of maize has distinctive hairs and strengthening cells. In *Rld* leaves an ectopic ligule develops on the abaxial leaf surface and epidermal cell types are displaced from the adaxial to the abaxial side (Freeling, 1992). In contrast to *lbl1*, the *Rld* mutation acts after leaf initiation.

2. Abaxial Determinants

KANADI1 (*KAN1*) and *KAN2* are functionally redundant as neither has a strong phenotype alone. In *kan1*, *kan2* double mutants, leaves are narrow and filamentous, and both leaves and floral organs are adaxialized (Eshed *et al.*, 2001; Kerstetter *et al.*, 2001). The KANADI proteins contain a highly conserved GARP (GOLDEN2, ARR B Class proteins Psr1) domain that is thought to be involved in transcriptional activation (Hall *et al.*, 1998; Sakai *et al.*, 1998; Kerstetter *et al.*, 2001). *KAN1* and *KAN2* are expressed throughout very young primordia and become abaxially localized shortly after *PHB* transcripts become adaxially localized (Eshed *et al.*, 2001; Kerstetter *et al.*, 2001).

Persistent uniform expression of *KAN1* and *KAN2* results in radial cotyledons and abaxialized leaves, and also results in loss of the vasculature in lateral organs (Eshed *et al.*, 2001; Kerstetter *et al.*, 2001). This suggests that abaxial identity is the ground state. Thus, activation of PHB-like proteins in response to a signal from the SAM may result in the down-regulation of *KANADI* genes, suppression of abaxial fate and specification of adaxial identity. This would suggest that *KANADI* genes are downstream of the *PHB*-like family. However, *PHV* and *REV* expression is more persistent in *kan1*, *kan2* double mutants (Eshed *et al.*, 2001).

kan1 and *kan2* were originally isolated as enhancers of *crabs claw* (*crc*), a floral mutation in a member of the YABBY family. There are five other *YABBY* genes in *Arabidopsis*, including *FILAMENTOUS FLOWER* (*FIL*) and *INNER-NO-OUTER* (*INO*), which also have mutant phenotypes involving loss of abaxial cell types and polarity in flowers, ovules and floral organs (Bowman and Smyth, 1999; Chen *et al.*, 1999; Eshed *et al.*, 1999; Siegfried *et al.*, 1999; Sawa *et al.*, 1999; Villanueva *et al.*, 1999). Alone, mutations in the *YABBY* genes have no vegetative phenotype, but in combination *fil* and *yab3* mutants reduce the differentiation between the adaxial and abaxial sides of the leaf. *YABBY* genes encode nuclear proteins with a zinc finger and a helix–loop–helix motif that resembles

the first two helices of the HMG DNA binding domain. *FIL, YAB2* and *YAB3* are expressed throughout young primordia but later become localized to the abaxial side. *CRC* and *INO* have similar expression patterns, but are expressed only in reproductive organs (Bowman and Smyth, 1999, Villanueva *et al.*, 1999). *YABBY* expression marks abaxial identity in mutants with altered polarity, but expression of *YABBY* genes is not sufficient for abaxial fate, as misexpression induces abaxial fate in only some cells (Siegfried *et al.*, 1999).

The YABBY family may be required for outgrowth. *kan1*, *kan2* double mutants have ectopic outgrowths on their adaxialized leaves reminiscent of *phan*. These outgrowths are abolished in a *kan1*, *kan2*, *yab3*, *fil* quadruple (Eshed *et al.*, 2001). The relationship between the *KANADI* genes and YABBY family is not simple. Overexpression of either family is epistatic to double mutants in the other: *35S:YAB3* is epistatic to *kan1*, *kan2*, but *35S:KAN 1/2* is epistatic to *fil yab3*. The *YABBY* and *KANADI* genes may act in parallel pathways as well as in series.

3. The Meristem Connection

The importance of communication from the meristem to the leaf for organ patterning has been demonstrated by surgical experiments (Sussex, 1954, 1955; Snow and Snow, 1959). Some of the mutants described above have meristem defects as well as polarity defects, suggesting that communication in the other direction is also important. For example, abaxialization of the leaf is associated with loss of meristem function. *PHAN* is not expressed in the meristem, but null alleles of *phan* can lead to SAM arrest (Waites *et al.*, 1998). Additionally, the most severely abaxialized *phan* leaves lack axillary meristems. Strong alleles of *lbl1* also lack a shoot meristem (Timmermans *et al.*, 1998). The adaxial domain may be required for signaling from the leaf to the meristem, promoting meristem function.

The adaxial side of the leaf is associated with meristematic competence. In dicots, axillary meristems are clonally related to the adaxial side of the leaf and can be thought of as dorsal structures (Irish and Sussex, 1992). Further, ectopic meristems on lateral organs consistently occur only on the adaxial leaf surface (Sinha *et al.*, 1993; Chuck *et al.*, 1996; Byrne *et al.*, 2000). Adaxilization of the leaf in *Phab1-D* homozygotes results in ectopic axillary meristems and *rev* mutants lose axillary meristems (Talbert *et al.*, 1995; McConnell and Barton, 1998; Otsuga *et al.*, 2001).

The influence of the adaxial leaf domain is not limited to axillary meristems. *Phab1-D* heterozygotes have meristems larger than wild type and partially rescue *stm-1* (McConnell and Barton, 1998). A role for *REV* in SAM function is suggested by double mutants with *argonaute* (*ago*). Strong *ago* mutants lack meristems and dorsoventral patterning, while weak alleles are adaxialized, although they lack axillary buds. *rev ago* double mutants have no cotyledons and no meristem. This phenotype suggests that *AGO* and *REV* act in separate but overlapping pathways controlling meristem function. (Kidner and Martienssen, in preparation)

AGO, a member of a large gene family conserved in animals and plants, is required for post-transcriptional transgene silencing by RNA interference, or RNAi (Fagard *et al.*, 2000). AGO shares the PAZ (Piwi, Argonaute, Zwille) domain with an RNAse helicase, DICER, thought to be responsible for degrading dsRNA (Bernstein *et al.*, 2001). Double mutant analysis indicates that *AGO* promotes abaxial and floral organ identity via RNAi and other gene silencing pathways (Kidner and Martienssen, in preparation). *AGO* is partially redundant with the closely related gene *PINHEAD/ZWILLE* (*PNH/ZLL*). However, while *AGO* is expressed uniformly in the young seedlings, PNH/ZLL is expressed on the adaxial side of the leaves and in the vasculature (Lynn *et al.*, 1999) in a pattern very similar to *PHAB* and *REV*. Like *ago* mutants, mutations in *PNH/ZLL* result in meristem defects. Double mutants do not accumulate STM protein, and a weak *stm* mutant enhances the dorsoventral defects in weak *ago* leaves (Lynn *et al.*, 1999; Kidner and Martienssen, in preparation). An intriguing possibility is that small RNAs, the product of RNAi, are the signal between meristems and leaves.

4. *The Origin of Polarity*

Vasculature is a highly polarized tissue of earlier origin than leaves (Gifford and Foster, 1989), and several of the genes required for dorsoventral patterning are also expressed in the vasculature, including the *PHB* family, the *KANADI* genes, and *PNH*. *PHB* family members have also been isolated from ferns (*Ceratopteris*; Aso *et al.*, 1999) and mosses (*Physcomitrella patens*; Sakakibara *et al.*, 2001), which are thought to have evolved leaves independently from the angiosperm lineage (Bateman, 1998). Therefore, these gene families are very ancient, and may have had an initial role in patterning vascular tissue in early non-leafy vascular plants, only later acquiring an additional function patterning lateral organs (Eshed *et al.*, 2001). However, neither *PHB* nor *PNH* have distinct polar expression within the vasculature.

McConnell *et al.* (2001) and Kersetter *et al.* (2001) suggest that the dorsoventral polarity of the leaf is derived from the peripheral/central polarity of the shoot. According to this view, the adaxial side of the leaf, the meristem and the vasculature are all related to the central part of the shoot, while the abaxial side of the leaf, non-vascular cells and non-meristematic cells are related to the peripheral region. This hypothesis is supported by the observation that in *35S::KAN* plants, when dorsoventral patterning is disturbed the vasculature is lost, as is meristem function. In addition, there is a gradient of *PHB* expression from the center of the embryo to its circumference and *PNH* is expressed in the central domain of the embryo, while *KAN1* is expressed in the peripheral cells. However, *KANADI* genes, *PHB*-like genes, and *YABBY* genes are initially expressed uniformly throughout the primordia, only becoming polar when the primordia is well separated from the meristem. Furthermore, *Phb1-D* does not cause the predicted ectopic vascularization.

According to Zimmerman's telome hypothesis, leaves are derived from branching shoots (Zimmermann, 1953). Dorsoventral patterning in the leaf could be related to dorsoventral patterning in the shoot. Anisophyllous stems, such as *Pilea* spp. and pea, are not radially symmetrical but dorsoventral (Dengler, 1999). Flowers are also modified shoots, and many flowers show pronounced dorsoventral asymmetry. The *cycloidea* (*cyc*) mutations of *Antirrhinum* abaxialize the flower causing dorsal petals to resemble lateral ones and lateral petals to resemble ventral ones. (Luo *et al.*, 1996; Cubas *et al.*, 1999). In contrast, *DIVARICATA* (*DIV*) is required for ventral petal identity (Almeida *et al.*, 1997). However, both *phan* and *handlebars* (*hb*) (which interacts with *phan*) act additively with *cyc* and *div* mutations in the floral meristem (Waites and Hudson, 2001). This suggests that dorsoventral patterning in lateral organs is at least partially independent of dorsoventral symmetry in the floral shoot.

It is not yet clear whether mechanisms specifying dorsoventrality will be universally applicable. In dicots, recessive mutations (in *YABBY* and *KANADI* genes) adaxialize the leaf, whereas, in monocots, recessive *lbl* mutations abaxialize the leaf. This suggests either that additional genes controlling dorsoventrality remain to be described in each species, or that the mechanisms of dorsoventrality are different in monocots and dicots. The second possibility is supported by the expression pattern of *YABBY* genes in maize. Two *YABBY* homologs closely related to *FIL* and *YAB3* have been isolated from maize. They are expressed on the adaxial side of the leaf primordia, rather than the abaxial side as in *Arabidopsis* (M. T. and Michelle Juarez, unpublished results). As monocot and dicot leaves have a common evolutionary origin (Gifford and Foster, 1989), this observation is reminiscent of reversal of dorsoventral polarity in sister animal groups within the bilateria. However, it is also possible that *YABBY* genes have different roles in the two branches of angiosperms.

V. THE CONTROL OF LEAF FORM

Leaves show a great diversity of form, some of which is clearly adaptive. For example, needles of pine trees growing in cold, dry climates limit transpiration, whereas compound leaves of palms withstand exposure to tropical storms. Most leaf forms do not have such clear adaptive roles. For example, the oaks of North America show a wide variety of leaf form, from the thin tapered leaves of willow oak to the broad leaves of the water oak and lobed leaves of white oak. Leaves can also vary in shape between juvenile and adult stages and under varying environmental conditions. Change in leaf shape through plant development, a phenomenon known as heteroblasty, has received a great deal of attention, and mutations affecting these transitions in *Arabidopsis* and maize have been isolated (Poethig, 1988; Dudley and Poethig, 1993; Conway and Poethig, 1997; Telfer *et al.*, 1997; Tsukaya *et al.*, 2000). This work is summarized in a number of recent reviews (Poethig, 1990; Kerstetter and Poethig, 1998).

A. MEDIOLATERAL EXPANSION OF LAMINA

Much of the diversity in leaf form arises from outgrowth of the leaf lamina in the mediolateral axis – from the midrib to the margins of the leaf. In monocots, the width of the leaf depends largely on lateral recruitment of founder cells from the meristem (Plate 2) and this requires dorsoventral patterning (Timmermans *et al.*, 1998). Patterning within the monocot meristem controls the fate of lateral leaf domains (Scanlon *et al.*, 1996). In dicots, lamina extension also requires dorsoventral patterning (Waites and Hudson, 1995), but it occurs well after the primordium is established (Plate 2). Although the dorsoventral boundary runs the length of the leaf blade, growth varies along the proximodistal axis: commonly growth is maximal in the middle of the leaf and restricted at the tip and at the base.

Mediolateral outgrowth along the petiole in *Arabidopsis* is affected by *LEAFY PETIOLE* (*LEP*), a member of the AP2/EREBP family of transcription factors (van der Graaff *et al.*, 2000). Overexpression of *LEP* results in a petiole with more lamina than wild type, and *LEP* may promote lamina outgrowth. Alternatively, *LEP* may convert the proximal petiole into more distal leaf blade. Two genes in tobacco, *lam1* and *fat*, are required for lamina outgrowth (McHale, 1992; 1993). Mature *lam1* leaves have severely reduced blades that lack adaxial cell types, reminiscent of *phan* mutants in *Antirrhinum*. However, *lam1* retains some dorsoventrality early in leaf development, suggesting that *LAM1* is not required for early patterning. Mosaic analysis shows that *LAM1* is required in the L3 to promote blade outgrowth and acts non-cell autonomously over a limited distance to rescue polarity defects in adjacent *lam1* layers (McHale and Marcotrigiano, 1998). Early leaf development in *fat* is also similar to wild type. After initiation, abnormal cell divisions result in leaves that are both narrower and thicker than wild type. Both *lam1* and *fat* probably maintain patterns established earlier in organogenesis.

Arabidopsis genes expressed at the leaf margins have been identified in gene- and enhancer-trap screens (Sundaresan *et al.*, 1995; Martienssen and Dolan, 1998). The outermost cell file of the early leaf primordia gives rise to epidermal cells at the very margins of the leaf (Poethig and Sussex, 1985; Tilney-Bassett, 1986). One gene expressed in L1 marginal cells is *PRESSED FLOWER* (*PRS*), which encodes a WUSCHEL-like homeobox gene. *PRS* is required for growth of the two lateral sepals as well as marginal growth in the medial sepals (Matsumoto and Okada, 2001). Lack of a vegetative phenotype suggests that *PRS* may be redundant in the vegetative phase.

B. CONTROL OF CELL DIVISION AND EXPANSION

Early studies in a wide range of species have revealed a detailed picture of the patterns of cell division, elongation and cell death that give rise to leaf form (Maksymowych, 1973). Clonal analysis, whereby genetically marked cells are

traced through development has been used to construct fate maps of the shoot meristem, as well as to investigate the action of genes on neighboring cells (Tilney-Bassett, 1986; Steeves and Sussex, 1989). For example, the size of clones marked in *Arabidopsis* seeds predicts there are 4–10 leaf founder cells in each cell-layer (Furner and Pumfrey, 1992; Irish and Sussex, 1992), but that founder cell recruitment continues after leaf initiation (Telfer and Poethig, 1994). Finally, mosaics of leaf shape mutants in cotton indicate that growth of one part of the leaf cannot compensate for lack of growth in another part, indicating that the shape of the leaf is locally controlled (Dolan and Poethig, 1991).

Clonal analysis of tobacco and maize established that cell divisions occur in the entire growing leaf and are not limited to the margins (Poethig and Sussex, 1985; Poethig and Szymkowiak, 1995). Histologically defined cell division patterns in *Arabidopsis* (Pyke *et al.*, 1991) have been refined using a cyclin∷GUS reporter (Donnelly *et al.*, 1999) as well as a PROLIFERA∷GFP genetrap reporters (Springer *et al.*, 2000).

Initially, divisions produce outgrowth along the proximodistal axis, forming a cylindrical peg. Subsequently, more divisions occur in the marginal regions, producing a lamina, and on the abaxial side causing the leaf to begin to curve over the SAM. Divisions cease in a proximodistal gradient starting with the distal tip of the leaf, and in a dorsoventral gradient starting in the abaxial mesophyll layer.

Changes in both cell division and cell expansion cause changes in organ size and shape. *ANT* is an AP2-domain transcription factor expressed in all lateral organs which regulates leaf and sepal size through control of cell number (Krizek, 1999; Mizukami and Fischer, 2000). In *ant* mutants, leaves are smaller than wild type and have fewer cells. On the other hand, overexpression of *ANT* results in larger organs with more cells (Krizek, 1999; Mizukami and Fischer, 2000). Changes in leaf growth can be induced by local application of expansions to developing leaf primordia, demonstrating that cell expansion is a major determinant of leaf shape (Reinhardt *et al.*, 1998; Pien *et al.*, 2001). *ERECTA* (*ER*) promotes elongation in leaves and internodes, and encodes a transmembrane receptor kinase with an extracellular leucine-rich repeat domain, indicating a role for cell–cell communication in the control of cell expansion and organ size (Torii *et al.*, 1996). *ROTUNDIFOLIA3* (*ROT3*), and *ANGUSTIFOLIA* (*AN*) act independently to control cell elongation along the proximodistal and mediolateral axis, respectively (Takahashi *et al.*, 1995; Tsuge *et al.*, 1996). *an* leaves are narrower than wild type, and *AN* encodes a CtBP/BARS protein which functionally interacts with a kinesin-like motor molecule (Turner and Crossley, 2001; Folkers *et al.*, 2002; Kim G. T. *et al.*, 2002). *ROT3* encodes a cytochrome P-450 and mutants have shorter leaves than wild-type (Kim *et al.*, 1998).

While cell division and expansion affect the size and shape of leaves, they are not required for patterning. In *tangled* (*tan*) maize mutants the orientation of cell-division planes along the longitudinal axis of the cell are aberrant, forming crooked or curved cell walls, but leaf shape is unaffected (Smith *et al.*, 1996). *tan* encodes a basic protein that associates with microtubules, and the defect in

cell division is correlated with misalignment of the cytoskeleton (Cleary and Smith, 1998; Smith *et al.*, 2001). Leaf shape is also maintained in maize *warty-1* mutants, although cell-division planes are abnormal and enlarged epidermal cells form warts on the leaf surface. Cells smaller than wild type are found adjacent to enlarged cells, suggesting a compensatory mechanism (Reynolds *et al.*, 1998). Cortical microtubular arrays are also disrupted in *ton* mutants, resulting in irregular cell expansion and disruption of cell division planes. However, cellular differentiation and organ patterning are not grossly affected (Traas, 1995). Finally, a dominant negative cell cycle regulator, cyclin-dependent kinase (Cdc2) leads to larger cells but normal plant morphology in tobacco (Hemerly *et al.*, 1995).

VI. DIFFERENTIATION

The differentiation of specific cell types is the final stage of leaf development. In common with other multicellular organisms, four main mechanisms have been proposed: neighbour induction, lateral inhibition (reaction/diffusion), signaling gradients and cell lineage (Wolpert, 1971). However, while there are examples of all these processes in animals, only neighbour induction, reaction/diffusion and some limited lineage effects have been identified in plants. These are discussed below.

A. DIFFERENTIATION OF GROUND TISSUES

1. The Development of the Vasculature

Venation patterns are richly diverse and form a basis for classification, particularly in fossil plants as they tend to be well preserved. Veins provide water and nutrients required by leaf cells, they transport photosynthate, and provide strengthening. The differentiation of many other cell types, including photosynthetic, supportive and epidermal cells, occurs in defined patterns relative to the vasculature, indicating that developing veins could provide the signals required for the surrounding pattern (Langdale *et al.*, 1989; Freeling and Lane, 1992). Alternatively, underlying patterns could direct the differentiation of both vascular and surrounding cells.

Leaves are divided into two groups depending on their vascularization (Zimmermann, 1953; Gifford and Foster, 1989). Microphylls, found in bryophytes and lycophytes, are thought to be derived from enations or outgrowths from the stem and have a single vascular trace. Megaphylls have more extensive vascularization and are accompanied by a ‘leaf gap’ in the vasculature of the stem. Megaphylls are thought to be derived from webbing between branches, and are characteristic of ferns, seed ferns and seed plants. However, a recent review suggests this division is artificial and there is in fact a continuum of vascularization patterns (Kaplan, 2001). Analysis of gene expression patterns may help resolve this issue.

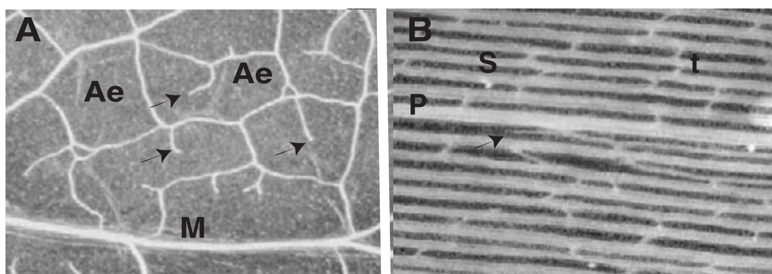

Fig. 2. Venation patterns in monocots and dicots. A. *Arabidopsis* has reticulate venation. A network of veins leads off from the midrib (M). The highest order of minor veins form blind endings in areoles (Ae) (arrows). B. Maize has parallel venation. The midrib and primary (P) and secondary (S) veins run longitudinally along the leaf. These veins develop in isolation, but anastomose near the blade sheath boundary and towards the leaf tip. Occasional veins maintain blind vein endings (arrow). Transverse veins (t) develop during the basipetal wave of differentiation and connect the parallel veins.

The veins of most dicots, including *Arabidopsis*, form a closed, continuous branching system comprised of a prominent midvein and several distinct orders of smaller veins (Candela *et al.*, 1999; Nelson and Dengler, 1997) (Fig. 2). The midvein develops first in continuity with the vasculature of the stem. Subsequently, the reticulate pattern of the higher-order veins differentiates. The last order of veins has a free end in the middle of an areole. It is unclear if vein formation simply stops in the middle of an areole, starts independently from the areole center, or whether provasculature cells may form a closed loop and differentiation proceed only half-way along. The latter is the case in *Trifolium* and in *Daphne pseudomezereum* (Hara, 1962; Lersten, 1965).

Although the ancestral monocot is thought to have had reticulate venation (Bharathan, 1996), modern monocot leaves typically have a parallel arrangement of longitudinal veins, which anastomose, or join, as the leaf narrows at each end. The midvein and major lateral veins initiate at the base of the leaf primordia in isolation from the vasculature of the stem. They extend acropetally into the leaf and basipetally to join the stem vasculature. As the number of cell files between existing veins increases, a new vein is initiated in the intervening space. As differentiation proceeds basipetally, small transverse veins develop forming connections between the longitudinal veins (Sharman, 1942; Nelson and Dengler, 1997) (Fig. 2).

Auxin appears to be a key player in vascular development. Auxin transport is known to be polar, and Sachs has suggested that the polar flow of auxin from the end of a vein into a differentiating field of cells, directs the formation of veins by ‘canalization’ (Sachs, 1981, 1991). Cells exposed to the highest levels of auxin develop as provascular cells, which in turn become polar transporters of auxin. Such a mechanism could explain the progressive development of the primary

and secondary veins of *Arabidopsis*. However, the formation in grasses of isolated parallel lateral veins that extend in both directions, as well as the simultaneous appearance of lower order veins in both monocots and dicots, is more difficult to explain by canalization.

An alternate hypothesis to explain the spatial patterning of veins is the reaction/diffusion model (Meinhardt, 1984). Slight variations in the concentration of a slow-diffusing activator and a fast-diffusing inhibitor can produce patterns of spots or stripes in an initially uniform undifferentiated field. In combination these can give rise to a reticulate pattern similar to that of dicot veins. Changes in the properties of the activator and inhibitor and the shape of the field can lead to the production of different patterns (Meinhardt, 1984). This model can explain the evenly spaced parallel venation patterns of grasses and the continual subdivision of expanding aereoles in dicot leaves. It can also explain the formation of free-vein endings in aereoles if these veinlets are actually initiated in the center of the aereole. However, this remains to be determined.

Arabidopsis plants grown on auxin transport inhibitors develop multiple midveins in isolation from the stem, and extensive vascularization at the leaf margins (Sieburth, 1999, Mattsson *et al.*, 1999). A requirement for auxin in vascularization is confirmed by the phenotype of mutants defective in auxin transport and/or signaling. These include *monopterous* (*mp*), *emb30/gnom, pin1, lopped1/tornedo1, bodenloss, short hypocotyl2*, and *auxin resistant6* (Mayer *et al.*, 1991; Carland and McHale, 1996; Cnops *et al.*, 2000; Hobbie *et al.*, 2000; Koizumi *et al.*, 2000; Vernoux *et al.*, 2000).

Strong *mp* alleles lack the basal portion of the embryo and are lethal to seedlings. *MP* belongs to the AUX/IAA family of transcription factors that bind to auxin response elements (Hardtke and Berleth, 1998). Weaker alleles have reduced auxin transport and unconnected vasculature, but have the normal complement of vascular cell types (Przemeck *et al.*, 1996). *MP* is initially expressed diffusely throughout the leaf, but becomes localized to the vasculature as it develops. This pattern is consistent with *MP* expression responding to a positive feedback loop directing vascular patterning (Hardtke and Berleth, 1998). The *cotyledon vascular patterning1* (*cvp1*), *cvp2, scarface* (*sfc*) and *vascular patterning1* (*van1*) through *van7* mutants all develop intact primary veins but discontinuous minor veins reminiscent of weak *mp* mutants (Carland *et al.*, 1999; Deyholos *et al.*, 2000; Koizumi *et al.*, 2000). The fact that defects in these mutations are limited to the minor veins could reflect differences in the genetic control of primary versus minor vein formation. Alternatively, development of minor veins could be more sensitive to subtle changes in the function of these genes. Consistent with the latter, *van7* was found to be a weak allele of *emb30/gnom* (Koizumi *et al.*, 2000).

The formation of discontinuous vasculature in these mutants is difficult to reconcile with the auxin canalization model for vascular patterning. Moreover, the discontinuous vascular segments form where minor veins normally develop,

suggesting that they do not affect the prepattern underlying the vasculature. This suggests that other signaling molecules are involved in vascular development. In fact, neither *cvp* mutant shows disturbances in the biosynthesis, perception or transport of auxin (Carland *et al.*, 1999). However, mutants affecting vascular spacing and minor vein initiation without affecting vascular differentiation have been isolated in the grass *Panicum maximum* (Fladung, 1994), while *midribless* mutants have also been identified in pearl millet, barley, oats and maize (Seip and Tschiya, 1979; Rao, 1989; Fladung *et al.*, 1991).

In *Arabidopsis* stems, vascular bundles differentiate xylem internally and phloem externally on the same radius. In the *amphivasal vascular bundle1* (*abv1*) mutant the phloem of each vascular strand is completely surrounded by a ring of xylem (Zhong *et al.*, 1999). Vascular patterns in *abv1* leaves were normal, although the arrangement of the vascular bundles there is also amphivasal. Vascular phenotypes of dorsoventral patterning mutants show that patterning within the vascular bundle is dependent on the dorsoventral axis. In *phan* mutants, phloem surrounds xylem in the adaxialized leaves, and in *Phab1-d* mutants only xylem forms. Two weak alleles of *REV* (*interfascicular fiberless* (*ifl*)*-1* and *ifl-2*) fail to differentiate secondary xylem and fibers in the stems (Zhong and Ye, 1999). In both mutants the xylem forms on the peripheral side of the vascular bundle, which may be related to the abaxial side of the leaf (Kerstetter *et al.*, 2001).

2. *Development of Photosynthetic Tissues*

The internal tissues of angiosperm leaves are adapted to maximize photosynthesis and respond to light, temperature, water stress and salt stress. In dicots, the adaxial side of the leaf has tightly packed columnar mesophyll cells forming the palisade layer, which is optimized for capturing sunlight. The abaxial side of the leaf has loosely packed mesophyll cells and more epidermal stomata in accordance with a greater role in gas exchange. High light levels (520 $\mu M\ m^{-2}\ s^{-1}$) result in elongated palisade cells and square spongy mesophyll cells. Low light levels (80 $\mu M\ m^{-2}\ s^{-1}$) give rise to palisade cells almost square in transverse section, and squamose spongy mesophyll. Defects in hormone signaling can have similar effects (Pyke and Lopez-Juez, 1999). *Arabidopsis* also has a distinct bundle sheath cell layer, consisting of a ring of elongated cells, surrounding vascular strands. The role of the bundle sheath in this case is unknown (Kinsman and Pyke, 1998).

In model dicots a number of genes affect mesophyll development, especially palisade cells. *PALECRESS* (*PAC*) in *Arabidopsis, DEFECTIVE CHLOROPLASTS AND LEAVES* (*DCL*) in tomato and *DIFFERENTIATION AND GREENING* (*DAG*) in *Antirrhinum*, are all required for normal palisade layer formation (Reiter *et al.*, 1994; Chatterjee *et al.*, 1996; Keddie *et al.*, 1996) *dcl* and *pac* mutants have spongy mesophyll cells throughout the leaf, while palisade cells in *dag* fail to fully expand. Chloroplast development is perturbed in all

three mutants and the wild type gene products are localized in the chloroplast. This suggests that signals from the chloroplast direct the final stages of palisade cell division and expansion. At least some of these signals are likely to be secondary metabolites. *CAB GENE UNDEREXPRESSED 1* (*CUE1*) encodes a phosphenolpyruvate/phosphate translocator required for phenolic metabolism, and mutants disrupt palisade cell development but can be rescued with aromatic compounds (Li *et al.*, 1995; Streatfield *et al.*, 1999). Likewise, tobacco plants overexpressing a myb domain transcription factor (*AmMYB308*) are impaired in phenolic acid metabolism and monolignol biosynthesis and have defects in palisade cells (Tamagnone *et al.*, 1998). In both mutants, leaves are reticulate because cells surrounding the veins are relatively dark green. A similar pattern is seen in mutants of *DIFFERENTIAL DEVELOPMENT OF VASCULAR ASSOCIATE CELLS* (*DOV*), which affect mesophyll but not bundle sheath chloroplasts in *Arabidopsis* (Kinsman and Pyke, 1998).

The C4 photosynthetic pathway is usually accompanied by two distinct chloroplast types, with and without granal stacks, confined to mesophyll and bundle sheath cells respectively. Mesophyll cells are loosely arranged between vascular strands, whereas bundle sheath cells are closely packed around the vasculature. This specialized Kranz anatomy allows for the spatial separation of CO_2 fixation. Fixation into a C4 acid occurs in the mesophyll, the C4 acids are shuttled into bundle sheath cells, where they are decarboxylated and the resulting CO_2 is fixed by RuBisCO. C4 photosynthesis is not strictly dependent on the Kranz anatomy. One member of the Chenopodiaceae has been found to carry out C4 photosynthesis in a single cell type, although in this case all photosynthetic cells have two types of chloroplast (Voznesenskaya *et al.*, 2001). An alternative method for concentrating CO_2 is Crassulacean acid metabolism (CAM). CAM is similar to C4 photosynthesis, except that the formation of C4 acids is temporally, not spatially, separated from decarboxylation and refixation of CO_2. Hence, CAM plants lack the specialized anatomy characteristic of C4 plants.

In maize it has been proposed that photosynthesis follows a C3 pathway by default and C4 specialization is achieved by both positional cues and a light responsive signal from the vasculature (Langdale *et al.*, 1988). The differentiation state of a cell is therefore determined by the distance from a vein. Cells directly adjacent to veins are derived from a procambial lineage and develop as C4 bundle sheath. Cells adjacent to the bundle sheath develop as C4 mesophyll, while cells more distant from the vein develop a C3 photosynthetic program.

Although position effects on cell fate are clear, in some cases position-independent cell lineage may impact on final cell identity. In the *tan1* mutant, cell-division patterns in the leaf are disrupted such that, late in leaf development, additional divisions of bundle sheath cells occur (Smith *et al.*, 1996; Jankovsky *et al.*, 2001). The progeny of these divisions, although more than one cell layer from a vein, develop as bundle sheath cells. One interpretation of this phenotype is that cell lineage determines the identity of these additional bundle sheath cells.

Mutations in maize demonstrate that separate genetic pathways specify differential development of C4 bundle sheath and mesophyll cells. Mutations in *Golden2* (*G2*) disrupt chloroplast development in C4 bundle sheath and C3 mesophyll, but not C4 mesophyll cells (Langdale and Kidner, 1994; Hall *et al.*, 1998). G2 is a transcription factor with relatives, *G2-like* (*Glk*) genes, identified in maize and rice. The expression pattern of *Glk* genes in rice, a C3 species, and in maize suggests these genes act redundantly in C3 development and have evolved specialized functions in C4 development (Rossini *et al.*, 2001).

B. DIFFERENTIATION OF THE EPIDERMIS

1. Patterning and Differentiation of Stomata

Stomata are specialized pores in the epidermis that allow gas exchange. The arrangement of stomata depends on the environmental conditions, and presumably reflects a balance between enabling photosynthesis and limiting transpiration (Cutter, 1978). Plants adapted to arid areas often have stomata grouped in protected pockets, whereas aquatic plants may lack stomata altogether (Bell, 1991). Also, many plants develop more stomata on the abaxial (shaded) side of the leaf, although this is generally not the case in the grasses.

Stomata are not randomly distributed on the leaf, in that directly adjacent stomata are rarely observed (Esau, 1977). Stomatal complexes consist of two guard cells flanking the pore and up to six subsidiary cells surrounding the guard cells. Stomatal development in dicots begins with an asymmetric cell division of the meristemoid mother cell (MMC) in which the smaller of the two daughter cells becomes the stomata initial or meristemoid. Subsequently the meristemoid may function directly as the guard mother cell (GMC) and divide once to give rise to the guard cell pair. Stomatal patterning in this case is established by regulating the frequency, spacing and/or orientation of the initial asymmetric division (Cutter, 1978).

Alternatively, the stomatal initial may divide to produce one or more non-stomatal cells before forming the GMC (Serna and Fenoll, 1997; Zhao and Sack, 1999). In *Arabidopsis*, each meristemoid divides 1–3 times before forming the GMC, so that their daughters comprise three-quarters of all epidermal cells. The divisions of the MMC and meristemoid are always orientated to separate adjacent meristemoids and GMCs. This pattern of cell division seems to be directed by cell–cell communication rather than by defined patterns of cell divisions within a cell lineage, as meristemoids, GMCs or guard cells affect the pattern of cell divisions in adjacent meristemoids (Geisler *et al.*, 2000).

How the fate of pavement cells, subsidiary cells and guard cells is specified in the correct position is unclear. In the *Arabidopsis* mutant *too many mouths* (*tmm*) divisions of the MMC are not orientated, but are random, giving rise to clusters of stomata (Yang and Sack, 1995). Geisler *et al.* (2000) suggest that *TMM* is required for signaling between differentiating meristemoids and adjacent MMCs to orient the asymmetric division. The *fourlips* (*flp*) mutant

produces stomata with one or two extra guard cells. Stomatal development in the *flp* mutant is normal until the GMC stage, but the GMC may divide more than once. Therefore, *FLP* either delays guard-cell fate or regulates the number of divisions of the GMC (Yang and Sack, 1995).

Distinct genetic pathways control the formation of stomata in different parts of the plant. *flp* and *tmm* have variable affects on stomata in different areas of the shoot (Geisler *et al.*, 1998). The patterning of stomata on the hypocotyl is controlled by *TRANSPARENT TESTA GLABRA* (*TTG*) and *GLABRAI* (*GL1*), which are also required for patterning of root hairs and trichomes (Berger *et al.*, 1998a). Stomatal development on the carpel requires the MADS (MCM-1, AG, DEFICENS, SRF) box gene *FRUITFULL* (Gu *et al.*, 1998).

In monocots, stomata are arranged in linear cell files. Stomatal spacing within a file occurs because each cell divides asymmetrically to produce a small apical stomatal initial and a basal interstomatal cell (Sylvester *et al.*, 1996). However, the underlying mechanism that determines which cell files will give rise to stomata is unknown. Charlton (1988) proposed that cells within a particular cell file may be synchronized in their cell cycle because they are clonally related, and that formation of stomata may depend on the cell cycle stage of a cell file at a specific point during leaf development. Consistent with this hypothesis, in *Tradescantia*, stomata development within a cell file is nearly synchronized (Chin, 1995). However, clonal analysis of the maize epidermis showed that linear files of stomata are not necessarily clonally related (Hernandez *et al.*, 1999). Stomata in the *Arabidopsis* hypocotyl also develop in linear cell files but, like root hairs, only in those files that overlie the anticlinal cortical cell walls (Berger *et al.*, 1998b). Patterning of stomata in maize could also be specified by signals from the underlying tissues, as no stomata develop in the cell files overlying the vasculature (Freeling and Lane, 1992). Alternatively, stomata development may be induced by signals that are transduced down files of cells rather than between cell files.

In dicot species that develop stomatal complexes with subsidiary cells, the subsidiary cells are usually derived from the meristemoid prior to GMC formation. The stomatal complex thus represents a single lineage unit. In contrast, subsidiary cells in grasses form following an asymmetric division of epidermal cells adjacent to the GMC (the subsidiary mother cells, SMC). The two SMCs become highly polarized and their nuclei become localized at the cell walls flanking the GMC (Stebbins and Shah, 1960). Following mitosis, the SMC divide asymmetrically to give rise to a small subsidiary cell flanking the GMC and a larger non-stomatal cell. Several mutants have been isolated that affect the asymmetric cell division of the SMC (Gallagher and Smith, 1999; 2000). *brick1* (*brk1*) and *pangloss* (*pan1*) are required for the polarized localization of the SMC nucleus, whereas *discordia1* (*dcd1*) and *dcd2* are required subsequently to guide phragmoplast and cell wall formation (Gallagher and Smith, 2000). Analysis of these mutants suggests that subsidiary cell fate may be specified by the asymmetric localization of determinants to the cell wall adjacent

to the GMC, which become localized to the inner daughter nucleus following mitosis. In *brk1* and *pan1* neither daughter cell usually acquires subsidiary cell fate, whereas in the *dcd* mutants one of the daughter cells does acquire subsidiary cell fate, but the size, shape or position of this cell is abnormal (Gallagher and Smith, 2000).

2. Patterning and Differentiation of Trichomes

Many plants have epidermal-derived hairs on the leaf surface. These hairs perform several roles. They can help control the plant's temperature or prevent water loss (e.g. *Atriplex*), they can secrete salt (e.g. saltbush), produce oils (e.g. mint) or toxins (e.g. stinging nettle), or discourage pests physically as is the case in *Arabidopsis* (Johnson, 1975; Esau, 1977; Khan *et al.*, 1986). Trichomes can be of commercial importance. The cotton fiber is a trichome produced on the seed coat, and the juice sacs of citrus fruits are trichomes on the adaxial surface of the ovary wall.

Arabidopsis trichomes are typical of the Brassicacea; single, large (up to 500 μm) branched, living cells that are highly polyploid. They are supported by a ring of socket cells which are not clonally related (Larkin *et al.*, 1996). Trichomes presumably induce the differentiation of neighboring epidermal cells into socket cells. Because *Arabidopsis* trichomes are non-essential, they offer a wonderful system for genetic analysis. Numerous trichome mutants have been isolated, most of which affect trichome morphogenesis (Marks, 1994; Hulskamp *et al.*, 1994).

Trichome development can be divided into two phases (Hulskamp, 2000). During the first phase, the incipient trichome undergoes endoreduplication and branches are initiated. The number of trichome branches is in part determined by cellular DNA content. Decreased DNA content has been correlated with smaller cell size and reduced branch number, and, conversely, increased DNA content can result in additional branches (Koornneef *et al.*, 1982; Perazza *et al.*, 1999). Trichome outgrowth and branch initiation are also affected by mutations or drugs that disrupt microtubule organization (Mathur *et al.*, 1999; Szymanski *et al.*, 1999; Hulskamp *et al.*, 2000; Folkers *et al.*, 2002). Furthermore, mutations in *ZWICHEL*, which encodes a kinesin-like motor protein, affect trichome branching (Oppenheimer *et al.*, 1997). Together these observations suggest an important role for microtubule-directed transport in early trichome development. The second phase of trichome development is characterized by a rapid cell elongation and vacuolization. These processes are affected following treatment with actin inhibitors and in a class of mutants known as *distorted*, which affect the organization of the actin cytoskeleton. Actin is initially diffusely distributed, but becomes localized in long stretched cables during this second phase of trichome growth (Szymanski *et al.*, 1999; Mathur and Chua, 2000). The requirement for different cytoskeletal proteins suggests that the mode of cell expansion varies in different stages of trichome development.

Four genes have been identified as having roles in creating the evenly spaced pattern of trichomes on the leaf. During leaf expansion, trichome initiation is sequential, with new trichomes initiating in the enlarging spaces between older trichomes. The timing of this process is governed by the *REDUCED TRICHOME NUMBER* (*RTN*) gene (Larkin *et al.*, 1996). *rtn* mutants cease to initiate new trichomes at an earlier stage of development than in wild type. Trichomes are initiated in a basipetal wave and each trichome is initially separated by 3–4 pavement cells. Maintenance of distance between initiating trichomes suggests a lateral inhibition model of patterning, similar to that proposed for phyllotaxy and vascular patterning. The three remaining patterning mutants have been proposed as candidates for two local activators, *GLI* and *TTG*, and one long-range inhibitor, *TRIPTYCHON* (*TRY*) (Hulskamp *et al.*, 1999).

GL1 encodes a myb domain protein required for trichome formation in all the aerial portions of the plant except for the leaf margins (Herman and Marks, 1989). *GL1* is initially expressed throughout the leaf epidermis but, following trichome initiation, expression becomes limited to developing trichomes (Oppenheimer *et al.*, 1991). Mosaic analysis has revealed that *GL1* acts in a cell autonomous or local manner (Hulskamp *et al.*, 1994). Like *gl1* mutants, *ttg* mutations also result in loss of all but the marginal trichomes of leaves (Koornneef, 1981). *TTG* encodes a WD40 repeat protein but, unlike *GL1*, expression of *TTG* is not limited to the epidermis (Walker *et al.*, 1999).

The *TRY* gene appears to be required to suppress trichome formation, as *try* mutants develop small clusters of trichomes (Schnittger *et al.*, 1999). Complex dose-dependent genetic interactions between *gl1*, *ttg* and *try* are consistent with these genes functioning via a lateral inhibition pathway to regulate trichome spacing (Oppenheimer *et al.*, 1991; Larkin, 1994; Schnittger *et al.*, 1996, 1998; Szymanski *et al.*, 1998). The cloning and expression pattern of *TRY* should establish whether the reaction/diffusion model holds for this system.

VII. PROSPECTS

Although the evolution of leaves was critical to the adaptive success of early land plants, leaves are thought to have evolved at least six separate times, occurring independently in mosses, liverworts, lycopods, ferns and sphenopsids as well as seed plants (Cronk, 2001). The molecular description of leaf development in land plants will reveal relationships between different leaf forms. Early descriptive and surgical experiments have been surprisingly accurate in predicting the mechanistic interactions emerging from model angiosperms, but it is molecular genetics that promises to revolutionize our understanding of these basic questions throughout the Plant Kingdom.

Important observations to emerge over the last few years include the prevalence of redundant, or partially redundant, gene families that regulate organogenesis in vegetative, floral and embryonic contexts. This may reflect the

evolution of flowering seed plants by gene duplication and divergence. It also has practical implications, in that multiple mutations are required to uncover phenotypes. Forward genetics and screening for enhancers of weak phenotypes has revealed some of these redundant factors (Eshed *et al.*, 1999; Byrne *et al.*, 2002). Reverse genetics will also allow comprehensive identification of multi-factorial regulatory systems. To aid functional genomics, large collections have been made of induced mutations and natural varieties of *Arabidopsis*, maize and rice.

Clonal analysis has previously demonstrated that cell–cell communication is critical for patterning in plants, but the direct transfer of transcription factors between cells was unanticipated until a few years ago. The control of plasmodesmatal function is thus an important factor in selective trafficking of transcription factors, and GFP fusions are providing a dynamic picture of this process during development (Kim *et al.*, 2001, Nakajima *et al.*, 2001; Kim J. Y. *et al.*, 2002). The trafficking of RNA molecules through the phloem (Balachandran *et al.*, 1997) suggests that coordination of plant development at the organism level could be via nucleotide messages as well as by phytohormones.

Lastly, epigenetic regulators are being recognized as important for development in plants, whose stem cell populations can last for thousands of years, and still retain the ability to give rise to determined derivative states. Cellular memory of gene expression patterns is imparted by chromatin modification in animal development, and a role in plants is suggested by the increasing number of developmental genes required for gene silencing. Interestingly, RNAi, which was first discovered as a mechanism for gene silencing in plants, has more profound implications for plant as opposed to animal development (at least outside the germline) (Bohmert *et al.*, 1998; Jacobsen *et al.*, 1999; Tabara *et al.*, 1999), while mutants in DNA-based silencing mechanisms, such as DNA methylation, are lethal in mammals but not in plants (Vongs *et al.*, 1993).

This is a particularly exciting time for model plant genetics. The sequence of entire genomes of *Arabidopsis* and rice has greatly accelerated plant research. Large collections of cDNA, knockouts, enhancer and gene traps and GAL4 promoter lines provide a wonderful range of resources, and genome databases such as TAIR, TIGR, GARNET and MATDB make the information and seed lines accessible to all. The last five years have witnessed enormous contributions to our knowledge of leaf development. The next review in this series on leaf development will likely address the development of microphylls, the evolution of various compound leaf forms and the genes responsible for the different developmental patterns of monocot and dicot leaves. We look forward to reading it.

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A Model for the Evolution and Genesis of the Pseudotetraploid *Arabidopsis thaliana* Genome

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ABSTRACT

The major part of the *Arabidopsis thaliana* genome is composed of a patchwork of duplicated chromosomal segments. We have built a model which, based on ancestral Brassicaceae, suggests an evolutionary pathway for the genesis of the modern *Arabidopsis* species through a series of successive independent chromosomal rearrangements. Translocations and inversions are thought to have produced a pseudotetraploid genome which functions as a true diploid, but with a larger proteome. Similar duplicated segments were also observed in *Brassica* spp., indicating that the segmental duplications predate the divergence of the two genera. The process of continuous duplications of segments, resulting in their translocation to new chromosomal locations in random direct or inverted centromere-to-telomere positions, appears as a significant evolutionary force.

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This has also been described in yeast and human genomes. This process resulted in an increase in genome size and created redundancy which was counterbalanced by a high frequency of single gene erasing.

I. INTRODUCTION

Genome duplication is believed to have played an important role during evolution (Ohno, 1970; Wendel, 2000), enabling genes to make evolutionary experiments (Kimura, 1983). The first evidence of duplications of plant chromosomes came from cytological studies, and genetic maps identified duplicated segments (Helentjaris *et al.*, 1988). Later on it was demonstrated both that duplicated loci mapped on to different chromosomes (McGrawth *et al.*, 1993), and that islands of conserved blocks exhibit parallel gene organization in *Arabidopsis* (Kowalski *et al.*, 1994). Data from plants reveal single gene duplications, duplications of conserved blocks, duplications of single chromosomes (i.e. aneuploidy) and duplications of entire genomes. The sequencing of the *Arabidopsis* nuclear genome (Lin *et al.*, 1999; Mayer *et al.*, 1999; Salanoubat *et al.*, 2000; Tabata *et al.*, 2000; Theologis *et al.*, 2000) revealed that most of the sequence is arranged in duplicated segments (The AGI, 2000; Blanc *et al.*, 2000; Paterson *et al.*, 2000; Vision *et al.*, 2000), similarly to the 55 duplicated segments of yeast (Wolfe and Shields, 1997). The emerging picture is that the *A. thaliana* (hereafter *At*) genome is organized with greater complexity than previously anticipated.

The question asked here is: how did the duplications of large segments arise in the *At* genome? It has been postulated that a single polyploidy event, associated with chromosomal rearrangements, generated a tetraploid genome (The AGI, 2000; Blanc *et al.*, 2000; Paterson *et al.*, 2000; Lynch and Conery, 2000; Wolfe, 2001).

Three possible explanations account for the present organization of the *At* genome. The auto- and allo-tetraploidization mechanisms postulate both a single duplication event of the whole genome and various rearrangements among and within chromosomes, resulting in the breaking of the duplicated chromosomes into smaller duplicated segments. A single duplication of an entire genome has important consequences, which can be tested. For example, a molecular clock analysis, i.e. dating of various different gene pairs, should converge on a single estimate of the age of the duplication event. However, the distribution of date estimates from *Arabidopsis* is heterogeneous (Lynch and Conery, 2000). Therefore an alternative and equally valid hypothesis could be that of numerous independent, successive duplications of chromosome segments, combined with intrachromosomal and interchromosomal rearrangements.

It is often assumed that tetraploidization (i.e. duplicating the whole gene set simultaneously), is a more parsimonious explanation for an increase in gene number than multiple independent events, but it has been shown by Hughes *et al.* (2001) that this is not necessarily true.

II. MOLECULAR PHYLOGENY OF *ARABIDOPSIS*

Molecular approaches have dramatically increased our current knowledge about the systematics of the genus *Arabidopsis*. *Arabidopsis*, a member of the tribe Arabideae, has now been placed in its appropriate phylogenetic context (Koch *et al.*, 1999, 2000). In this paper we use x for the basic expected chromosome number of the lineage, and $2n$ for the chromosome number in somatic cells, regardless of the ploidy level ($2n = 2x$, $2n = 3x$, $2n = 4x$, …). The phylogenetic data show that the genus *Arabidopsis* is polyphyletic, consisting of several lineages (Koch *et al.*, 1999). The closest relatives of the self-pollinating *A. thaliana* ($2n = 2x = 10$) include outcrossing species with $2n = 2x = 16$, namely *A. lyrata* (= *A. petrea*) and *A. halleri* (formerly *Cardaminopsis*). The polyphyletic *Arabis* genus ($2n = 2x = 14$) is more distant (Koch *et al.*, 2000). Most polyploid species are polyphyletic, having originated from genetically divergent parents (Soltis and Soltis, 2000). The *A. thaliana* ecotypes diverged 1.5 million years (Ma), ago, and there is evidence that *A. thaliana* experienced a rapid population expansion (Koch *et al.*, 2000; Kuittinen and Aguade, 2000). Indeed, *A. thaliana* is found in various environmental milieux, from subtropical areas to the Himalayas. The last common ancestor of *A. thaliana* and its nearest relatives (e.g. *A. lyrata*) appeared approximately 5 Ma ago (Kuittinen and Aguadé, 2000). The genus *Capsella* is related to some *Arabis* and to the *At* lineage (Koch *et al.*, 2000). The lineages leading to *A. thaliana* and *Capsella rubella* ($2n = 2x = 16$) diverged about 8 Ma ago, and the synteny between these two species is more pronounced than the synteny between *A. thaliana* and *Brassica* (Cavell *et al.*, 1998; Acarkan *et al.*, 2000). Nevertheless, many *Brassica* genes show a high level of nucleotide sequence conservation with their *A. thaliana* orthologues (Cavell *et al.*, 1998). *A. thaliana* diverged from *Arabis* clades 10 Ma ago, and from *Brassica* roughly 15 to 19 Ma ago (Acarkan *et al.*, 2000; Yang *et al.*, 1999). Congruent fossil and molecular evidence suggests that early Cruciferae appeared about 30 Ma ago (Koch *et al.*, 2000), whereas the divergence for Magnoliids (including monocots) and Eudicots (including Brassicaceae) is estimated to have occurred about 200 Ma ago (Ku *et al.*, 2000).

The *Arabidopsis* phylogenetic trees produced by Koch *et al.* (1999 and 2000) do not provide the necessary evidence supporting the hypothesis of a genome duplication either from $n = 8$ to $n = 16$, or from two successive rounds of genome duplications (from $n = 8$ to $n = 16$ to $n = 32$). On the contrary, the trees suggest that the Brassicaceae ancestor might possess either $n = 8$ or $n = 4$ chromosomes.

To support the 2R hypothesis (i.e. two successive rounds of polyploidization) suggested by Ku *et al.* (2000) and Vision *et al.* (2000), the phylogenetic trees would additionally have to be used to test the significance of a 4:1 orthological relationship with an outgroup (Wolfe, 2001). The characteristic features of such trees are a 2 + 2 specific topology, described as (AB)(CD), as well as identical

ages shared by the first and second divergences. None of the four-member families or four-member clusters in the *At* serine/threonine kinases (about 1000) showed such typical topologies consistent with multiple rounds of whole genome duplications.

III. THE EVOLUTION OF THE *ARABIDOPSIS* GENOME

On Plate 1a we present a picture of the *At* genome, based on 34 duplicated segments, including the thirteen identified previously (Blanc *et al.*, 2000), the four additional segments evidenced by the *Arabidopsis* Genome Initiative (The AGI, 2000), the five segments (Blanc *et al.*, 2000) split into 16 smaller blocks (The AGI, 2000), and the small block identified recently by Tavares *et al.* (2000). Other additional small blocks might be present (see at http://mips.gsf.de/proj/thal/db/gv/rv/rv_frame.html).

The features of the segments and the small blocks indicated in Plate 1a suggest that *At* is a 'pseudotetraploid'. The subtelomeric regions from most of the *At* chromosomes possess duplicated segments suggestive of duplication from one chromosome to another by non-reciprocal translocations. The resolution of subtelomeric duplicated segments such as Nor1–2, RAQ1–2, a1–2, s1–2, t1–2, u1–2, z1–2, ae1–2, revealed additional subtelomeric duplicated segments: x1–2, y1–2, i1–2, j1–2, k1–2. The latter observation was used in the elaboration of the proposed model, as well as a reduced number of chromosomal rearrangements. A careful analysis of the metacentric chromosome I revealed a very different segmental organization compared to the other chromosomes, since the segments c, e, f, g and h are observed on both arms. Plate 1b shows the chromosomal organization of a putative *Arabidopsis* ancestor.

Most of the duplicated segments are separated from each other by 'orphan' segments, suggesting independent evolutionary events. For 18 out of the 34 duplicated segments, the orientation with respect to the centromere to telomere orientation is the same in each copy. Such a random orientation of the duplicated segments indicated that inversions were involved.

The model depicted on Plate 2 illustrates a putative evolutionary derivation of *Arabidopsis* chromosome segments and blocks. Segments have been duplicated from ancestral chromosomes through successive chromosomal rearrangements, producing the present *A. thaliana* genome. The organization of the *At* chromosomes is presented as having derived from 45 chromosomal rearrangements involving mostly non-reciprocal translocations and inversions. Several large segments were split into smaller blocks, namely c–e–f–g–h, B–l–m, j–k–w, n–o–p–q–r, x–y–z and ab–ac–ad. Rearrangements include the formation of one isochromosome, the duplication of one interstitial segment, three reciprocal translocations, 16 non-reciprocal translocations, 22 paracentric and two pericentric inversions. Inversions and translocations are thought to be the main form of large-scale rearrangement of the *At* genome.

Chromosomal rearrangements often produce segregational distortions, therefore, only part of the chromosomal rearrangements have been maintained during further evolution.

Present-day data suggest that at least part of the segments might have been duplicated at different times. Ancient steps in the duplication of *At* segments are numbered 1 to 8 in Plate 2. Isochromosome formation (e.g. chromosome I) is postulated to be one of the earliest events in the evolutionary history of the *Arabidopsis* chromosomes.

Events from 9 to 36 putatively occurred in the *Arabido-Brassica* ancestor. Indeed, comparative genomics has identified synteny among related Brassicaceae. Duplicated segments identical to those detailed in *Arabidopsis* are observed within the genomes of the ancestor of Brassica sp. (Bancroft, 2000). They are pointed out in Plate 2 by the events 9, 23 and 29. This unambiguously demonstrates that the occurrence of the above duplications predates the divergence of the two species, dated to 15–20 Ma ago (Yang *et al.*, 1999; Acarkan *et al.*, 2000). This was confirmed by the fact that segments j and k (event 9) are also duplicated in *Capsella rubella* (Rossberg *et al.*, 2001). Richards *et al.* (1991) described centromeric telomere-similar sequences on chromosome I, with an additional copy in the central region of chromosome V. These are remnants of chromosome rearrangements (translocation, inversion) consistent with the events 19 and 21 shown on Plate 2.

Our model suggests that recently duplicated segments correspond with events 41 to 45. This includes the small blocks RAQ (Tavares *et al.*, 2000) and NOR (nucleolar organizing region). Although species related to *At*, namely, *C. rubella*, *A. halleri* and *A. lyrata*, possess different types of nuclear rDNA ITS (internal transcribed spacer), *At* possesses only one ITS type (Koch *et al.*, 1999). This is in accordance with the recent duplication of the nucleolar organizer region indicated by event 42 in Plate 2. Moreover, a recent inversion event has relocated centromeric sequences to an interstitial location, on the short arm of chromosome IV (Fransz *et al.*, 2000), which corresponds with the event 37 on Plate 2.

The relative age-distribution of gene duplicates obtained by Lynch and Conery (2000), suggested that there are very ancient duplicated genes, ancient ones and more recent ones. Some of the gene duplicates are older than the divergence between *Arabidopsis* and *Brassica*. Part of the distribution from Lynch and Conery (2000), i.e. the secondary peak on their fig. 2, is consistent both with a single duplication event under the assumption that the number of silent substitutions does not increase linearly with time, and with successive events over a period of a few million years – both occurring between 30 and 65 Ma ago, depending on the molecular clock used. The Lynch and Conery (2000) data concern only part of the *At* genes, and exclude the genes that identified more than five matching sequences using a blast analysis, hence excluding large families (e.g. kinases occupied 4% of the gene set). Unfortunately, The AGI (2000) shows that the proportion of proteins which belong to members of families of

five or more, is much higher in *At* (i.e. 41%) than in any other species (i.e. 9.1 to 25.6%). Similarly, recent data from yeast suggested both ancient and recent duplications (Friedman and Hughes, 2001a). These data are consistent with a continuous process generating duplications of segments, with a fragmentation of large segments, and with the fact that chromosomal rearrangements play a significant role in genome changes (Song *et al.*, 1995).

IV. SEGMENTAL DUPLICATIONS

Proir to the *Arabidopsis* sequencing data, analyses of the yeast genome showed evidence of duplicated chromosomal blocks. Hence the structure of the *Saccharomyces cerevisiae* genome was interpreted to be a palaeopolyploid (Wolfe and Shields, 1997). Other experimental data indicated that such chromosomal segments might result from a reiteration of independent segmental duplications of chromosomal blocks (Coissac *et al.*, 1997; Llorente *et al.*, 2000a,b).

Figure 1 illustrates 34 duplicated blocks, and in between are located 67 unique blocks or blocks at chromosome termini. This indicates that the probability of being copied is 0.25. Based on a Poisson approximation, we obtained the expected distribution of segments: 105 unique, 26 duplicated and four triplicated or more. A chi-squared test compares observed and expected numbers, resulting in a one degree of freedom chi-squared = 5.28. This has a probability $p = 0.02$, suggesting that the difference between the expected and observed number is significant.

The main criticism of the hypothesis of successive duplication of segments is the lack of triplicated or tetraplicated segments. Such an argument is directed against chromosomal rearrangements, either producing successive duplications of segments in a diploid genome, or creating duplicated segments in a tetraploid genome. In the latter hypothesis, it is suggested that inter- and intra-chromosomal rearrangements broke up entire duplicated chromosomes into smaller segments. Complete reshuffling of the *At* genome after a tetraploidization process also requires translocations and inversions. The main difference is that deleted or translocated chromosomes are inherited in one-quarter of the diploid progeny, instead of one-sixteenth in a rediploidized tetraploid. Our careful studies of multigenic families from the *At* genome, have provided evidence for two triplicated and one quadruplicated blocks. Similarly, results from Winge *et al.* (2000) suggested a pentaplicated block. In any case, these blocks are quite short, including at the most 25 genes. Data from Vision *et al.* (2000) described many overlapping segments. First, a reduced size is expected after recombination events which occur randomly across non-duplicated or previously duplicated segments. Second, extensive deletion of genes and significant reshuffling, after a triplication event, would obscure the evidence that a triplication has occurred.

Another hypothesis, more and more supported by experimental data, particularly in human and primate genomes, is suggested. The fact that the duplicated

segments do not overlap is compatible with a model of successive independent duplication processes using non-random hot-spot recombination sites. An increasing number of human diseases result from DNA rearrangements involving strand exchanges at recombination hot-spots (Stankiewicz and Lupski, 2002). Most of the identified recombination promoting sequences (Emanuel and Shaikh, 2001) are produced by non-allelic homologous recombinations between region-specific low-copy repeats (Lupski, 1998). Systematic analyses of the putative unstable genomic parts might identify such regions in *Arabidopsis*. Anyway, there is evidence of various types of recombination hot-spots. First, AT-rich sequences of the human genome might be prone to recombination events that lead to rearrangements (Shaffer and Lupski, 2000). Similarly, the CAGGG repeats and α-satellite are involved in the duplication mechanism (Crosier *et al.*, 2002). Second, other regions of the genome commonly undergo recombination between highly homologous sequences. Recombination events between copies of a duplicated gene were shown in yeast by Ryu *et al.* (1998). Many of the recently duplicated segments in primates are located in regions that are hot-spots for chromosomal instability (Samonte and Eichler, 2002). The pericentromeric and subtelomeric chromosome regions show a significant excess in the location of the segmental duplications. Alternatively, duplicated segments provide templates for non-allelic homologous recombination events (Samonte and Eichler, 2002). There is evidence that genome rearrangements often are non-random events, but rather reflect genome architectural features that promote non-allelic homologous recombinations (Stankiewicz and Lupski, 2002).

Although most examples of duplicated segments from *Arabidopsis* show synteny, sometimes the duplicated genes are outnumbered by interspersed orphan genes. Recent data from Fischer *et al.* (2001) show evidence of a new process of gene erasing: the accumulation of numerous mutations in the coding sequence of one copy created ‘relics’. Macrosynteny has also been observed among chromosome segments from *At* and soybean, *At* and tomato, and, to a lesser extent, *At* and rice (Devos *et al.*, 1999; van Dodeweerd *et al.*, 1999; Grant *et al.*, 2000; Ku *et al.*, 2000; Liu *et al.*, 2001).

Most of the duplicated segments from *At* are also duplicated within *Brassica* and *Capsella*, but they were not observed to be duplicated in soybean, tomato and rice genomes. This indicates that most of the duplications of segments have occurred between the emergence of early Brassicaceae and late Eurosids (i.e. 15–90 Ma ago). The consistency of this model should be tested by further studies. Firstly, the proposed hypothesis for the structural evolution of the *At* genome remains to be examined using comparative genomics, with regard to chromosome organization and colinearity, in related species such as *A. petrea*, *A. halleri* and *C. rubella*. Secondly, the molecular mechanisms underlying the rearrangements might have required DNA double-strand breaks, with or without the use of particular sequences for recombination. The latter should likewise be tested.

V. THE *ARABIDO-BRASSICA* ANCESTOR

The organization of the present-day genomes of *Arabidopsis* and *Brassica* suggests very different histories of speciation. This is exemplified by the genome of *Brassica nigra*, which consists of three rearranged copies of an ancestral genome (Lagercrantz and Lydiate, 1996), exhibiting disomic segregation. *Brassicae* have undergone a process by which genome size increases by cyclic allopolyploidization (Truco *et al.*, 1996; Lagercrantz, 1998), followed by structural changes. This corroborates the hypothesis of accelerated chromosome evolution among progenies of newly formed polyploids (Acarkan *et al.*, 2000). In contrast *Arabidopsis* has maintained a reduced chromosome number and a small genome, containing numerous duplicated segments. Approximately 90 rearrangements took place since *Arabidopsis* and *Brassica* diverged (Acarkan *et al.*, 2000). This is consistent with the chromosomal rearrangement events detailed in Plate 2.

If indeed the *Arabidopsis* ancestral genome had experienced polyploidization events through multiple duplications of chromosome segments, then the present chromosome complement of $2n = 2x = 10$ suggests a chromosomal history with an increase from $n = 4$ to $n = 5$, rather than a decrease from $n = 8$ to $n = 5$ through chromosomal centric fusions as suggested previously (Koch *et al.*, 1999, 2000). The hypothesis that chromosome numbers smaller than $n = 8$ (i.e. $n = 5$, 6 or 7) were derived from $n = 8$ (Koch *et al.*, 2000), leads to several consequences. Such a reduction from $n = 8$ to $n = 5$ suggests a previous duplication of the whole ancestral genome from $n = 4$ to $n = 8$, and also requires an ancestor with $n = 8$ chromosomes providing *Capsella* ($n = 8$) with an *At*-like chromosome IV separated into two chromosomes (Schmidt *et al.*, 2001). The latter fits well with the fact that chromosome IV from *At* corresponds to two linkage groups in *Capsella* (Schmidt *et al.*, 2001). Moreover, such an organization would indeed be consistent with the evolutionary derivation of the *At* genome from successive duplications of segments, as indicated in Plate 2, followed by a break of chromosome IV into two telocentrics. On the contrary, a tetraploid *Arabidopsis thaliana* ancestor possessing eight telomeric (i.e. only a long arm: L, with a terminal centromere) haploid chromosomes in the form AL AL BL BL CL CL DL DL, will not easily explain the chromosomal organization of the present *At* (AS–AL, BS–BL, CS–CL, DS–DL, ES–El) through centric fusions. This requires that extensive chromosomal reorganizations took place during *Arabidopsis* speciation, a scenario established for the separation of yeast lineages (Piskur, 2001).

VI. CONCLUSION

Genome duplications have undoubtedly occurred in the plant kingdom (e.g. alfalfa, banana, cotton, maize, sugar cane, sunflower, tobacco), mostly via allopolyploidization, and sometimes in several instances (rape, wheat). A plethora of data support the hypothesis that the *Arabidopsis* genome has duplicated at least partially:

(1) conserved gene order and orientation between duplicated segments;
(2) phylogenetic support for a 2:1 orthology relationship with an outgroup; and
(3) synteny between related species (*Arabidopsis*, *Capsella*, *Brassica*).

The key event in the production of a diploid through auto- or allo-polyploidization processes is the switch from multivalents in meiosis to bivalents (disomic segregation). The autopolyploidization hypothesis failed to explain the management of a redundancy for 18 000 genes. A high sequence identity among duplicated chromosomes promotes misalignment during meiosis, and induces recombination between paralogous instead of allelic loci. Aberrant meiosis produces quadrivalents, trivalents and monovalents, chromosome bridges and 'laggards', each leading to chromosome losses or breakage in gametic cells. Allopolyploid plants have developed particular genetic controls, such as the Ph gene in wheat (Okamoto, 1957), in order to avoid pairing between homologous chromosomes.

The segmental duplications identified in *A. thaliana*, compared with those observed for the human genome (Venter *et al.*, 2001), might reflect the unusual plasticity of a plant genome. Plasticity might explain the difficulties associated with inferring ancient polyploidization events. The question addressed in Fig. 1 was whether the chromosomal rearrangements observed in *At* were preceded by an ancestral tetraploidization event. We noticed that a tetraploidization event introduces a discrepancy between phylogenetic and molecular data. Indeed, it does not seem possible to support the view that the duplicated segments in *Arabidopsis* arose through a single whole genome duplication. The variation in the number of chromosomes (n = 5, 6, 7, 8, 16, 24) found in the phylogenetic tree (Koch *et al.*, 1999, 2000) suggests that chromosome duplications have been very dynamic events during the evolutionary history of *At*. The various elements discussed in this paper provide evidence that the hypothesis of successive chromosomal rearrangements and duplications of segments offers a more parsimonious explanation of the present *Arabidopsis* genome structure than the hypothesis of genomic duplication followed by successive chromosomal rearrangements. Evidence indicates that the events of duplication of segments may have occurred 30–40 Ma ago, before the divergence of *Arabidopsis* and *Brassica*, some probably even earlier, and others in recent times (Lynch and Conery, 2000). Our calculation showed an average rate of about three chromosomal rearrangements in 1 Ma, which requires the addition of new telomeres. Interestingly, it has been shown that yeast possesses a genetic control for telomere addition (Myung *et al.*, 2001).

Polyploidy or successive rounds of polyploidization were not expected for a plant possessing five chromosomes and a small genome. Due to numerous duplicated segments, the present *Arabidopsis* genome looks like a pseudotetraploid, functioning as a true diploid, but with a larger proteome than the hypothesized ancestor. According to the model of divergent resolution, an evolutionary consequence of gene duplication is speciation (Taylor *et al.*, 2001). Successive duplications of chromosome segments would have generated redundancy for a limited number of genes, offering an evolutionary capacity by the creation of paralogous

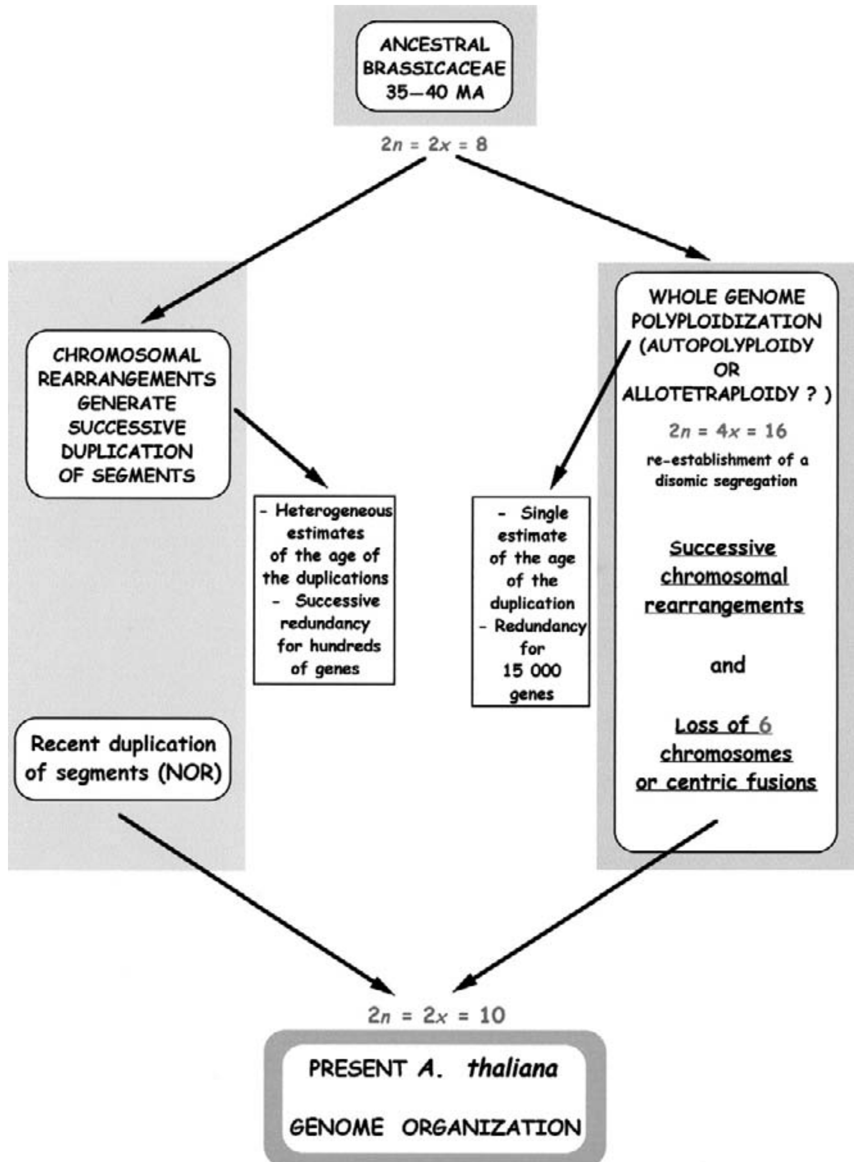

Fig. 1. Comparison of two alternative models for the origin of the present *Arabidopsis* genome structure.

genes. Our model provides the basis for studying both the number and age of successive events punctuating the history of the *Arabidopsis* ancestor. The model will also be useful to evaluate further the extent of genome synteny and organization between the Brassicaceae. In humans and in fish as well, the hypothesis that gene number increased as a result of multiple independent duplication

events appears more parsimonious than genome tetraploidization (Friedman and Hughes, 2001b; Robinson-Rechavi *et al.*, 2001).

GLOSSARY

Cytogenetic

Acrocentric: chromosome possessing a subterminal centromere

Allotetraploid: contains four genomes from two different ancestral parents that hybridize

Autotetraploid: contains four identical genome sets, by simple doubling of a single genome

Metacentric: chromosome possessing a centromere in a medial position and therefore with arm lengths almost identical.

Genomic

Colinearity: conservation of the gene order within a chromosome segment, between different species

Orthologue: homologous gene loci from two different species that originated from a common ancestor

Paralogue: homologous gene that originated by gene duplication in a defined organism

Syntheny: conservation in genome structure (gene order, gene orientation) within a chromosome segment from two species.

Intrachromosomal rearrangements include various cytogenetic changes, such as interstitial and terminal deletions, interstitial duplications, inversions and isochromosome formation, while interchromosomal rearrangements include various types of translocations.

Centric fusion: fusion of centromeres from two chromosomes having a terminal centromere (telocentric)

Deletion: loss of a distal or interstitial chromosome fragment

Duplication: Establishment of two copies of a chromosome fragment

Inversion: Reversal of a chromosome fragment within the chromosome (pericentric inversion includes the centromere)

Isochromosome: chromosome resulting from a duplication of a single chromosome arm

Translocation: reciprocal chromosome fragment exchange between two chromosomes (reciprocal translocation) or transfer of a single chromosome fragment from one chromosome to another (non-reciprocal translocation).

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Age-related Resistance to Plant Pathogens

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ABSTRACT

Plant tissues often show different levels of resistance to pathogens depending on their age. Some of the many types of age-related resistance (ARR) have similarity to known plant defence systems, including preformed defences, race-specific gene-for-gene resistance, systemic acquired resistance and induced systemic resistance. However, the molecular basis for the mature onset of ARR and the nature of the signalling pathways involved are poorly understood. The discovery of a novel form of ARR in the model plant *Arabidopsis* and the presence in tomato of homologous genes that provide either ARR or seedling resistance to leaf mould are likely to lead to an increased research effort that may address these questions. This review summarises examples of ARR and compares them with more thoroughly characterised forms of disease resistance with a view towards future research.

I. INTRODUCTION

A. WHAT IS AGE-RELATED RESISTANCE?

Many plants show changes in the resistance of whole plants or plant tissues to viral, bacterial, oomycete and fungal pathogens that correlate with stages of plant development. This phenomenon, known as age-related resistance (ARR), is most often seen as resistance that increases with plant or tissue age (Table I). Examples include the ‘adult-plant’ resistance of wheat, barley and maize to numerous rust fungi and the resistance of older tissues within the hypocotyl of soybean seedlings to *Phytophthora sojae* infection. In contrast, some plant tissues become more susceptible to disease with increasing age. Mature potato plants, and older leaves of *Schefflera arboricola* and onion plants, are more susceptible than younger plants or leaves to infection by *Phytophthora infestans*, *Pseudomonas cichorii* and *Alternaria porri*, respectively (Peterson and Mills, 1953; Miller, 1983; Chase, 1985).

B. THE POTENTIAL VALUE OF ARR TO PLANT BREEDERS

The presence of a dominant avirulence gene in the pathogen and a corresponding dominant resistance gene in the host plant are the prerequisites for race-specific gene-for-gene resistance (Flor, 1971). The activation of plant defences in race-specific resistance is thought to depend on the specific recognition of an avirulence gene product by a resistance gene product. Many resistance genes occur in multigene families and encode receptor-like proteins with amino acid residues in their receptor domains often encoded by codons that show evidence

TABLE I
Examples of age-related resistance to plant pathogens

Pathogen type	Pathogen	Host	Reference
VIRUSES	Cauliflower mosaic virus	Turnip	Leisner *et al.* (1992)
	Cauliflower mosaic virus	*Arabidopsis*	Leisner *et al.* (1993)
BACTERIA	*Pseudomonas syringae* pv. *tomato*	*Arabidopsis*	Kus *et al.* (2002)
	P. syringae pv. *maculicola*	*Arabidopsis*	Kus *et al.* (2002)
	Xanthomonas oryzae pv. *oryzae*	Rice	Mazzola *et al.* (1994)
OOMYCETES	*Peronospora tabacina*	Tobacco	Wyatt *et al.* (1991)
	Phytophthora capsici	Pepper[a]	Kim and Hwang (1989)
	Phytophthora parasitica	Tobacco	Hugot *et al.* (1999)
	Phytophthora sojae	Soybean	Lazarovits *et al.* (1980)
FUNGI	*Blumeria graminis* f. sp. *hordei*	Barley	Russell *et al.* (1975)
	Cladosporium fulvum	Tomato[a]	Panter *et al.* (2002)
	Colletotrichum lindemuthianum	Bean	Griffey and Leach (1965)
	Leptosphaeria maculans	Rapeseed[a]	Ballinger and Salisbury (1996)
	Magnaporthe grisea	Rice	Kahn and Libby (1957)
	Puccinia coronata f.sp. *avenae*	Oats[a]	Peturson (1944)
	Puccinia graminis f. sp. *tritici*	Wheat	Sunderwirth and Roelfs (1980)
	Puccinia sorghi	Maize	Headrick and Pataky (1987)
	Puccinia striiformis	Wheat	Quayoum and Line (1985)
	Puccinia triticina	Wheat[a]	Bartos *et al.* (1969)
	Pyrenophora teres	Barley[a]	Douiyssi *et al.* (1998)
	Rhizoctonia solani	Bean	Bateman and Lumsden (1964)
	Rhizoctonia solani	Cotton	Hunter *et al.* (1977)
	Uromyces vignae	Cowpea[a]	Heath (1993)

[a] Examples where race-specificity has been demonstrated

for diversifying selection (Dodds *et al*., 2000). Some avirulence gene products, or elicitors, including NIP1 (Rohe *et al*., 1995), AvrPto (Chang *et al*., 2000), ECP2 (Lauge *et al*., 1997, 1998a), AvrXa7, AvrXa10 and Avrxa5 (Bai *et al*., 2000) have been identified as proteins that increase the ability of pathogens to colonise plants in the absence of cognate resistance genes. Selection may favour mutations in elicitors that destroy or modulate avirulence function to avoid

detection by the plant. For example, races of *C. fulvum* with mutations that disrupt the *Avr9* gene (Marmeisse *et al.,* 1993) or reduce the stability of Avr4 (Joosten *et al*., 1997) can overcome specific resistance provided by the tomato *Cf-9* and *Cf-4* genes, respectively. Site-directed mutagenesis has demonstrated that a mutation can allow the AvrPto elicitor to retain its virulence function, but lose its avirulence function (Shan *et al*., 2000). Hence, mutations in avirulence genes that prevent the detection of elicitors by the plant may be favoured under the intense selection pressure imposed by deployment of a single resistance gene in a crop monoculture. This is one explanation why some forms of resistance provided by single resistance genes have been overcome by the epidemic appearance of new pathogen races (Roelfs, 1984).

Incorporating many genes for resistance to a single pathogen into a crop, or 'pyramiding', is a plant breeding objective that aims to increase the durability of resistance because, in theory, it increases the number of pathogen mutations needed to overcome race-specific resistance (reviewed in Line and Chen, 1995; Huang *et al*., 1997). Selection pressure caused by the 'pyramiding' strategy could potentially favour 'supervirulent' pathogens, so an alternative strategy involves planting fields with multiple lines of a crop plant, each expressing a different resistance gene (Zhu *et al*., 2000).

Natural 'pyramiding' may already have inadvertently contributed to the durability of some forms of disease resistance, including examples where mature plant resistance and seedling resistance have been expressed at the same time. The tomato *Cf-9* gene cluster provides race-specific resistance to leaf mould (*Cladosporium fulvum*). This cluster contains both the *Cf-9* seedling resistance gene (Jones *et al*., 1994) and the *Cf-9B* mature-plant resistance gene (Panter *et al*., 2002). *Cf-9*-mediated resistance has been effective in controlling leaf mold for many years despite the occurrence of races of *C. fulvum* virulent on seedlings carrying *Cf-9*. The durability of *Cf-9*-mediated resistance could, in part, have been due to the mature-onset disease resistance conferred by *Cf-9B* (Panter *et al*., 2002). This underlying form of ARR was probably not detected in the past because it has always been easier to handle seedlings rather than mature plants in resistance screens (Takken *et al*., 1999). This example emphasises the potential value of genes conferring ARR in providing an additional reservoir of genes to increase the durability of disease resistance.

C. CURRENT QUESTIONS ABOUT ARR

The onset of some forms of ARR correlates with flowering, while the onset of others correlates with plant age, leaf size, or the synthesis of secondary metabolites or defence proteins. Some forms of ARR may conform to the gene-for-gene model or be non-specific, may be salicylic-acid dependent or independent (Hugot *et al*., 1999; Kus *et al*., 2002), or may be based on inducible or preformed defences. Some types of ARR appear to utilise components of known signalling pathways including those that lead to race-specific resistance in

seedlings, induced systemic resistance, systemic acquired resistance, or leaf senescence, but at least one appears to involve a novel defence pathway (Kus *et al.*, 2002). It is not clear how many types of ARR there are, what developmental or environmental cues trigger their onset, or what signalling molecules, signal transduction pathways and effector molecules are involved. This review aims to summarise our current knowledge of selected examples of ARR with these questions in mind and to compare ARR with other forms of disease resistance, with a view towards future research.

II. EXAMPLES OF ARR

A. ARR TO VIRUSES

Wounding allows viruses to gain entry to plant cells and, after initial reproduction of viral particles in the infected host cells, infection can spread locally through plasmodesmata and systemically through the phloem (Agrios, 1997).

Turnip and *Arabidopsis* both show non-specific ARR to cauliflower mosaic virus (CaMV). Leisner *et al.* (1992) showed that CaMV was able to spread systemically and infect whole young turnip leaves, but only the base of leaves that were larger than one-third the size of fully mature leaves. Age-related resistance to CaMV has been studied in more detail in *Arabidopsis*, where it was found to be related to the onset of flowering (Leisner *et al.*, 1993). Some early-flowering *Arabidopsis* lines, including Ws-0, showed ARR to long-distance movement of CaMV that was established between 11 and 27 days after germination (Leisner *et al.*, 1993). This resistance could be compromised by delaying the flowering of these lines, suggesting that ARR to virus infection in early-flowering *Arabidopsis* was caused by a narrower 'window' of susceptibility to long-distance virus movement delimited by the floral developmental programme (Leisner *et al.*, 1993).

Tobacco shows race-specific ARR to tobacco mosaic virus (TMV). Older plants and older leaves of mature tobacco plants carrying the *N* gene for TMV resistance have been found to show smaller hypersensitive lesions than younger plants or leaves (Yalpani *et al.*, 1993). However, the appearance of lesions *per se* is a symptom of *N*-mediated resistance to TMV infection, suggesting that in this case increased resistance may have been caused by the enhancement of *N*-mediated seedling resistance with age, possibly by a weak, non-specific ARR.

B. ARR TO BACTERIA

Several species of the gram-negative bacterial genera *Pseudomonas* and *Xanthomonas* are pathogens that enter plants via wounds, hydathodes or stomata (Agrios, 1997). Bacterial cells multiply and spread in the apoplast until a critical

bacterial density is reached and intercellular 'quorum signalling' is then thought to induce the production of virulence factors that kill host cells, resulting in disease lesions (Huguet, 2000).

Some *Arabidopsis* ecotypes display mature-plant resistance to bacterial speck disease caused by *Pseudomonas syringae* pathovars. Resistance is characterised by reduced bacterial growth *in planta* and absence of disease lesions. Ten-fold fewer *P. syringae* pv. *tomato* DC3000 cells grew in Col-0 *Arabidopsis* plants inoculated 40 days after seed germination (DAG) than in those inoculated 30 DAG (Kus *et al.*, 2002). Plants were grown under short daylength conditions with a nine hour photoperiod, which would be expected to induce flowering 35 DAG (Pineiro and Coupland, 1998). Therefore, there was a good correlation between the onset of ARR and the vegetative–floral phase transition.

ARR was accompanied either by no macroscopic symptoms or by chlorosis of leaves on infected plants. ARR was also seen in the Ws-0 and B *Arabidopsis* ecotypes and in response to infection by *P. syringae* pv. *maculicola*, suggesting that it lacks the host or pathogen specificity characteristic of gene-for-gene interactions. ARR-like resistance of immature Col-0 plants was also observed following water or nutrient limitation, suggesting that ARR could also be induced by stress.

Despite a lack of ARR in *NahG, sid1* and *sid2* plants, which cannot accumulate salicylic acid (SA), there was no correlation between ARR and the presence or absence of an intact *NPR1* gene (Cao *et al.*, 1997), which encodes a signalling protein required for the transcriptional activation of pathogenesis-related PR proteins in response to SA during systemic acquired resistance, or the expression of PR-1 and PR-5, suggesting that ARR utilised a distinct SA-dependent signalling pathway and effector molecules different from those involved in systemic acquired resistance (Fig. 1). *NPR1* is also required for induced systemic resistance, along with *ETR1*, which encodes a protein involved in ethylene perception, but the presence of ARR in plants with mutations in *NPR1* and *ETR1* suggested that ARR was also distinct from induced systemic resistance (Fig. 1). Plants with the *pad3-1* and *eds7-1* mutations affecting camalexin synthesis and basal resistance, respectively, still exhibited ARR, suggesting that induced phytoalexin synthesis and basal resistance were not involved either. The leaf senescence marker *SAG-13* was not induced during ARR, suggesting that the induction of the leaf senescence pathway and the associated induction of defence-related (DR) proteins were not involved in ARR (Fig. 1). The exclusion of these various pathways points to the existence of a novel SA-dependent pathway for ARR in *Arabidopsis*. The further exclusion of a number of effector molecules associated with these pathways also points to the existence of novel effectors of ARR in *Arabidopsis*.

Mature *Arabidopsis* plants, but not seedlings, were able to produce an antibacterial molecule in the apoplast following infection by *P. syringae* pv. *tomato*. It has been suggested that antibiotic effector molecules, expressed only in mature pathogen-challenged plants, could limit bacterial population growth and

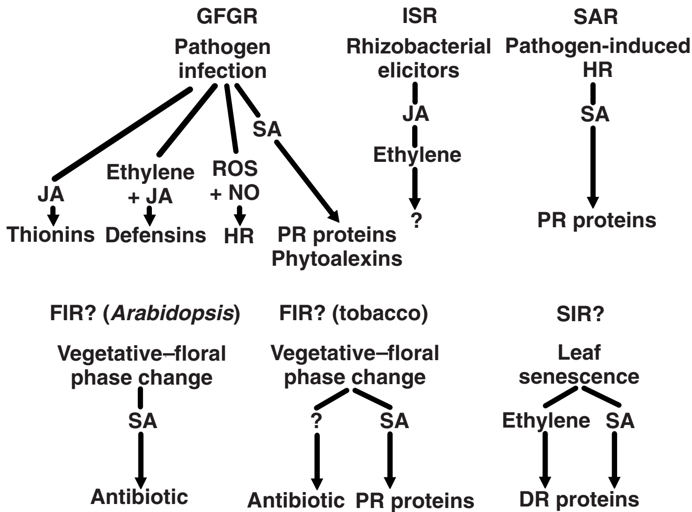

Fig. 1. Signalling pathways leading to different types of resistance. The gene-for-gene resistance (GFGR), induced systemic resistance (ISR) and systemic acquired resistance (SAR) pathways of *Arabidopsis*, and hypothetical senescence-induced resistance (SIR?) and flowering-induced resistance (FIR?) pathways are shown. These are characterised by their dependence, or lack thereof, on key signalling molecules. These are: jasmonic acid (JA), ethylene, salicylic acid (SA), reactive oxygen species (ROS) and nitric oxide (NO). Effector molecules produced by these defence pathways include thionins, defensins, pathogenesis-related (PR) proteins, phytoalexins, uncharacterised antibiotic molecules and defence-related (DR) proteins. The hypersensitive response (HR) refers to the programmed death of plant cells in infected tissue that restricts the growth of pathogens.

prevent ‘quorum sensing’ that would otherwise lead to the destruction of host cells (Kus *et al*., 2002).

Rice plants containing the *Xa21* resistance gene show race-specific ARR to bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae,* that develops between 11 and 21 days after seedling emergence (DAE) from the soil (Mazzola *et al*., 1994). ARR was manifest as a reduction in the length of disease lesions on the leaves of *Xa21* plants that correlated with increasing plant age. *Xa21* plants also supported lower numbers of bacteria than susceptible plants of the same age when inoculated between 21 and 41 DAE. However, when inoculated at 61 DAE, both lines supported fewer bacteria than did younger susceptible plants, presumably because of an additional form of non-specific ARR. A more detailed study of *Xa21* plants, where each leaf was inoculated as soon as it was fully expanded, showed that resistance gradually increased from leaf 2, which was fully susceptible, to leaf 9, which was fully resistant (Century *et al*., 1999). This suggested that resistance correlated with the stage of plant development at which a given leaf emerged, rather than leaf age *per se*.

C. ARR TO OOMYCETES

The oomycetes, which include the plant pathogenic genera *Phytophthora* and *Peronospora,* are fungus-like organisms that are more closely related to the brown algae than to the true fungi. Mycelia of *Phytophthora infestans*, the causal agent of potato late blight, produce sporangia that are released from the infected plant and germinate under cool, humid or wet weather conditions to release zoospores. These are chemotactically attracted to root surfaces of host plants, where they encyst and germinate. Germ tubes penetrate the cuticle directly or infect plants through wounds or natural openings. Once inside the plant, the hyphae parasitise host cells by forming haustoria that eventually kill these cells, resulting in disease lesions (Agrios, 1997). Other *Phytophthora* spp., which cause root and crown rot diseases and black shank of tobacco, and *Peronospora* spp., which cause downy mildew diseases and blue mould of tobacco, have a similar life cycle.

Tobacco plants show non-specific ARR to blue mould, caused by *Peronospora tabacina* (Reuveni *et al*., 1985; Wyatt *et al*., 1991; Wyatt and Kuc, 1992). Leaves on the main stem of tobacco plants develop resistance to blue mould when plants are between 10 and 12 weeks old, coincident with the onset of flowering. However, this resistance cannot be activated by the induction of premature flowering, and has been suggested to correlate with the onset of senescence in the oldest leaves rather than flowering (Wyatt *et al*., 1991; Wyatt and Kuc, 1992). Resistance correlates with a 2–2.5-fold increase in the basal activity levels of three potentially antimicrobial enzymes: a β-1-3 glucanase, a chitinase and a peroxidase (Wyatt *et al*., 1991). It is unclear whether the isoforms of these enzymes are the same as those induced during systemic acquired resistance, but this is a possibility.

Non-specific resistance of mature tobacco plants to black shank disease caused by *Phytophthora parasitica* also occurs at the early flowering stage, 10–12 weeks after germination, and results in a reduction in the size of disease lesions on leaves (Hugot *et al*., 1999). Inoculation experiments with *NahG* tobacco plants, which have little or no salicylic acid (SA), and studies of zoospore germination in intercellular fluids of vegetative and flowering tobacco plants indicated that two developmentally controlled signalling pathways contributed to ARR against *P. parasitica*. ARR was partially compromised by the *NahG* transgene, indicating that one defence component probably required the accumulation of SA coincident with flowering (Yalpani *et al*., 1993). However, SA-independent antimicrobial activity was observed in the intercellular washing fluids of mature *NahG* tobacco plants, but not seedlings (Hugot *et al*., 1999). This activity caused a 10-fold inhibition of cyst germination *in vitro* and could, in theory, have inhibited cyst germination *in vivo* (Hugot *et al*., 1999).

After infection with *Phytophthora sojae*, soybean hypocotyls show a gradient from susceptibility in the youngest tissue, which exhibits disease lesions, to asymptomatic resistance in the oldest tissue (Lazarovits *et al*., 1980). This non-

specific ARR correlates with more rapid induction of the phytoalexin glyceollin in older tissue than in younger tissue following infection (Ward *et al*., 1980). Glyceollin production was also induced in hypocotyls during race-specific resistance to *P. sojae*. Interestingly, older tissue within the soybean hypocotyl was more responsive than younger tissue in terms of glyceollin production to the elicitor of race-specific resistance, even though a gradient of resistance was not seen (Ward *et al*., 1980). These results suggest that some components of both the race-specific and non-specific pathways leading to resistance against *P. sojae* are under developmental control.

D. ARR TO FUNGI

As shown in Table I, there are many more observations of ARR to pathogenic fungi than to other types of plant pathogen. These include examples of both race-specific and non-specific ARR. Non-specific ARR to *Rhizoctonia solani* in bean has been attibuted to an age-related increase in the extracellular calcium ion concentration and its inhibitory effect on a fungal polygalacturonase (Bateman and Lumsden, 1964). Similarly, the formation of extraxylary fibres and lignified xylem vessels during plant development has been used to explain the non-specific ARR of bean plants to anthracnose caused by *Colletotrichum lindemuthianum* (Griffey and Leach, 1965). In these two cases, ARR precedes pathogen challenge and the defences are preformed.

As this review aims to focus on possible mechanisms for ARR, only one example of race-specific ARR induced by pathogen challenge and another of non-specific ARR are described in detail, namely, *Cf-9B*-mediated resistance to tomato leaf mould and *Sr2*-mediated resistance to wheat stem rust, respectively.

1. Cf-9B-Mediated Resistance to Tomato Leaf Mould

C. fulvum is a biotrophic ascomycete that infects susceptible tomato plants under conditions of very high humidity, causing leaf mould disease. Conidia germinate on the abaxial surfaces of tomato leaves and hyphae gain entry to the plant via stomata on the third or fourth day after inoculation (Langford, 1937; De Wit, 1977). The fungus forms an extensive network of hyphae in the intercellular spaces by nine days after inoculation and aerial hyphae emerge approximately four days later on the abaxial leaf surfaces and form conidia (Langford, 1937; Bond, 1938; De Wit, 1977; Lazarovits and Higgins, 1975; Hammond-Kosack and Jones, 1993).

The onset of resistance provided by the *Cf-9B* gene occurs between the early flowering and early fruiting stages of plant development (Parniske *et al*., 1997; Lauge *et al*., 1998b; Panter *et al*., 2002). Leaves of mature *Cf-9B* plants lack patches of aerial hyphae that are seen on susceptible Cf0 plants two to three weeks after inoculation, and instead have pale, chlorotic lesions of approximately 0.5 cm in diameter. When leaves of mature *Cf-9B* plants are stained with

trypan blue and examined microscopically, these lesions are found to correspond with areas of extensive hyphal colonisation of the mesophyll with little, if any, conidiation. However, leaves of fruiting Cf0 plants show smaller and less extensive patches of aerial hyphae and conidiation than vegetative Cf0 plants, suggesting that genetically susceptible plants have weak, non-specific ARR that could also contribute to the resistance of mature *Cf-9B* plants.

2. Sr2-Mediated Resistance to Wheat Stem Rust

The asexual life cycles of cereal rust fungi (*Puccinia* spp.) begin with the germination of urediospores on cereal leaves and the growth of germ tubes across leaf surfaces towards stomata (Staples and Macko, 1984). Upon reaching a stoma, an appressorium develops and penetration ensues. Once penetration has been achieved, a substomatal vesicle is formed, which differentiates into hyphae that ramify through the leaf tissue and parasitise individual plant cells by forming haustoria (Staples and Macko, 1984). In a well-established infection, the mycelium gives rise to emergent sporogenous cells that differentiate into urediospores (Harder, 1984).

The *Sr2* gene provides mature-plant resistance to wheat stem rust (*Puccinia graminis* f. sp. *tritici*) that becomes active between the second-node stage and anthesis of wheat plants (Sunderwirth and Roelfs, 1980). At anthesis, ARR in four wheat cultivars containing *Sr2* was manifest as a reduced number of uredia formed per plant, a less severe infection type and a different distribution of lesions within the plant (Sunderwirth and Roelfs, 1980) in comparison with two susceptible cultivars. *Sr2*-mediated resistance appears to be effective against all known races of *P. graminis* f. sp. *tritici* (Knott, 1968; Knott, 1971), with only slight variation in the resistance phenotype caused by plant genetic background (Sunderwirth and Roelfs, 1980). The *Sr2* gene has not yet been cloned and the molecular basis for this type of ARR is not yet understood.

III. WHAT CONTROLS THE ONSET OF ARR?

A. THE ONSET OF RACE-SPECIFIC ARR

Mature-plant resistance could, in theory, be caused by the induction of resistance gene transcripts following developmental cues. The *Pib* (Wang *et al.*, 1999), *Xa1* (Yoshimura *et al.*, 1998) and $Hs1^{pro1}$ (Cai *et al.*, 1997) genes for rice blast, rice blight and beet nematode resistance, respectively, were found to be transcriptionally upregulated during infection or parasitism. The *Pib* and *Xa1* genes were also induced by other stimuli, suggesting that their induction could be a consequence of the inoculation process rather than infection *per se*. *Pib* transcripts were induced by spraying plants with water or by maintaining plants in darkness for 24 hours (Wang *et al.*, 1999). *Xa1* transcripts were induced by wounding (Yoshimura *et al.*, 1998). The upregulation of resistance genes may prepare the plant for an enhanced response in conditions that could

favour infection. Wounding of plant tissues may remove physical barriers to infection, such as a waxy leaf cuticle, and high environmental humidity could favour fungal spore germination or the survival of bacterial cells on the plant surface. Inducible expression of these genes could minimise the resources being diverted from plant growth and reproduction at times when there is less risk of infection. Similarly, genes conferring mature-plant resistance could be induced by developmental cues, such as the elevation of salicylic acid (SA) levels in tobacco plants at the vegetative–floral stage transition (Yalpani *et al.*, 1993).

A study of *Xa21* transcription involving gene-specific reverse transcriptase-PCR (RT-PCR) and RNAse protection assays aimed to test whether the mature onset of resistance in rice to bacterial blight was caused by a change in the activity of the *Xa21* promoter during plant development (Century *et al.*, 1999). However, *Xa21* was found to be transcribed at a low level and was not induced by wounding, pathogen inoculation or developmental cues (Century *et al.*, 1999). *Cf-9* provides seedling and mature-plant resistance to tomato leaf mould, while *Cf-9B* protects only mature plants (Jones *et al.*, 1994; Panter *et al.*, 2002) and the promoter regions of these two genes differ by small insertions and deletions and a region of low sequence similarity (Panter *et al.*, 2002). Hence, differences in the ability of these promoters to bind transcription factors could have caused *Cf-9B* and *Cf-9* to respond differently to developmental signals. However, the analysis of promoter-*gusA* fusions in tomato plants and gene-specific RT-PCR suggested that *Cf-9* and *Cf-9B* were both expressed in seedlings (Panter *et al.*, 2002). Furthermore, a *Cf-9* promoter–*Cf-9B* coding region swap did not protect tomato seedlings from infection by leaf mould with *Cf-9B* specificity, although it did protect mature plants, and the reciprocal swap protected seedlings with *Cf-9* specificity. Taken together, these results suggested that the mature onset of resistance mediated by *Xa21* and *Cf-9B* was not controlled by the activation of their respective promoters during plant development. However, this does not exclude the possibility that resistance genes providing ARR could be controlled at the post-transcriptional level in response to a developmental cue (Century *et al.*, 1999) or that other components of defence signalling could be under developmental control. An alternative reason for the mature onset of race-specific ARR could be an increase in the *in planta* stability of elicitors, coincident with a particular stage in plant development.

B. THE ONSET OF NON-SPECIFIC ARR

The onset of non-specific forms of ARR has been variously correlated with the vegetative–floral transition or the start of senescence in the oldest leaves of developing plants, and in some cases the triggering of other forms of resistance. Signals leading to, or following, these events may trigger the onset of ARR and this possibility is discussed in the next two sections.

IV. SIMILARITIES BETWEEN ARR AND OTHER FORMS OF PLANT DEFENCE

A. SYSTEMIC ACQUIRED RESISTANCE

Systemic acquired resistance (SAR) provides long-lasting broad-spectrum disease-resistance to secondary infection 24 hours or more after a primary infection that resulted in a hypersensitive response (HR), or the treatment of plants with SA or an SA analogue (reviewed in Barker, 2000). The onset of SAR is dependent on the production of SA throughout the plant and correlates with the induction of a characteristic fingerprint of pathogenesis-related (PR) proteins in tobacco and *Arabidopsis* (Ward *et al*., 1991; Uknes *et al*., 1992). The long-distance signal contributing to SAR remains unknown, but SA is thought to potentiate SAR by 'priming' cells for a rapid HR upon secondary infection and by inducing the production of PR proteins, some of which have been shown to have antimicrobial properties *in vitro* (Fig. 1).

The onset of the form (or forms) of ARR that protect tobacco against blue mold and black shank disease correlates with an increase in the production of SA and the PR-1a, PR-1b and PR-1c proteins in tobacco leaves (Fraser, 1981, Uknes *et al*., 1993; Wyatt *et al*., 1991; Yalpani *et al*., 1993; Hugot *et al*., 1999). ARR to blue mould also correlates with increases in the basal activities of glucanase, chitinase and peroxidase activities (Wyatt *et al*., 1991). However, no clear cause-and-effect relationship between the induction of these various PR proteins and enzymes and resistance to blue mould and black shank disease was found in the literature. The protection of plants from more than one pathogen and the production of SA and PR proteins suggest that this form (or these forms) of ARR could be similar to SAR. The removal of inflorescences and old leaves from flowering plants dramatically reduces the production of PR proteins in remaining leaves (Fraser *et al*., 1981), so a signal originating from these plant tissues could potentially take the place of a primary pathogen infection in triggering ARR, as opposed to SAR. Nevertheless, there is also an SA-independent component of ARR to black shank disease in tobacco (Hugot *et al*., 1999).

Other forms of ARR do not resemble SAR. Although age-related resistance of *Arabidopsis* to bacterial speck was found to be dependent on the accumulation of SA, this form of ARR did not require NPR1, which is required for SAR, and was not correlated with the expression of the PR-1 and PR-5, which are effectors for SAR in *Arabidopsis* (Kus *et al*., 2002). This suggests that the form of ARR that protects *Arabidopsis* from bacterial speck is distinct from SAR.

B. RHIZOBACTERIA-MEDIATED INDUCED SYSTEMIC RESISTANCE

Rhizobacteria-mediated induced systemic resistance (ISR) is a form of broad-spectrum resistance that is triggered by lipopolysaccharides, siderophores or SA that are produced by non-pathogenic root-colonising bacteria (Fig. 1) (see a

review in Van Loon *et al*., 1998). ISR in *Arabidopsis* does not require an increase in SA, jasmonic acid or ethylene production by the plant or correlate with the induction of proteins transcriptionally activated by these signalling molecules in other defence responses (see a review in Van Loon *et al*., 1998; Van Wees *et al*., 1999). However, the induction of the jasmonic acid-responsive *Atvsp* gene after pathogen inoculation is enhanced in plants exhibiting ISR (Van Wees *et al*., 1999). *Atvsp* is homologous to genes encoding vegetative storage protein acid phosphatases in soybean and its role in plant defence is poorly understood (Berger *et al*., 1995). In *Arabidopsis*, ISR requires sequential jasmonic acid and ethylene signalling steps, but does not require upregulation of the production of either of these molecules, or surprisingly, a functional NPR1 signalling protein (see a review in Parker, 2000). Presumably, NPR1 interacts with different upstream and downstream signalling partners in ISR or is present in a different oligomeric signalling complex, in comparison to SAR. The ability of plants to exhibit ISR has been mapped to the *ISR1* locus in *Arabidopsis*, which controls the response to ethylene (Pieterse *et al*., 1998; Ton *et al*., 1999; Ton *et al*., 2001).

ARR to bacterial speck in *Arabidopsis* does not require an intact NPR1 signalling protein or the ETR1 ethylene receptor, suggesting that it is distinct from ISR (Kus *et al*., 2002). In addition, the Ws-0 *Arabidopsis* ecotype, which lacks *ISR1*, displays ARR to bacterial speck (Ton *et al*., 1999; Kus *et al*., 2002). To date, there is no strong evidence to suggest that any form of ARR is related to ISR, although this remains a possibility.

C. SENESCENCE

Senescence has been described as a form of programmed tissue death in which nutrients are recovered from dying leaves. A set of plant proteins called defence-related (DR) proteins (Quirino *et al*., 2000), some of which bear a similarity to PR proteins, are induced during leaf senescence in *Brassica napus* and *Arabidopsis* plants (Buchanan-Wollaston, 1994; Hanfrey *et al*., 1996; Quirino *et al*., 1999). In *Arabidopsis*, there are subsets of DR proteins that are induced in an SA-independent, partially SA-dependent, or completely SA-dependent manner during leaf senescence (Quirino *et al*., 1999; Morris *et al*., 2000). The subset of DR proteins whose induction depends on SA also require PAD4, a protein required for race-specific defence responses triggered by a subclass of resistance proteins (Glazebrook, 2001), or both PAD4 and NPR1 for their induction (Morris *et al*., 2000). If DR proteins have antimicrobial properties, their expression in senescent leaves could be a strategy to prevent opportunistic pathogen infections (see a review in Quirino *et al*., 2000). *Arabidopsis* plants with the *ein2* mutation, which inhibits ethylene perception, or with the senescence-specific *ore1* and *ore9* mutations, show delayed leaf senescence and presumably, delayed expression of DR proteins (for a review see Quirino *et al*., 2000). It would be interesting to find out whether similar mutations delay the onset of

ARR in pathosystems, such as the tobacco–blue mould system, where there appears to be a correlation between leaf senescence and ARR.

A higher level of resistance in older leaves than in younger leaves of mature plants might implicate senescence in ARR. This has been observed in ARR to oat crown rust (Peturson, 1944), wheat leaf rust (Pretorius *et al*., 1988), rice blast (Roumen *et al*., 1992), rice bacterial blight (Koch and Mew, 1991), and tobacco mosaic virus (Yalpani *et al*., 1993). Older leaves of wheat plants also appear to have non-specific resistance to stem rust, in addition to ARR provided by the *Sr2* resistance gene (Sunderwirth and Roelfs, 1980). A correlation between the onset of ARR to blue mould and the senescence of older leaves, rather than the vegetative–floral transition in tobacco plants (Wyatt *et al*., 1991; Wyatt and Kuc, 1992), provides indirect evidence for the role of senescence in ARR.

There are also examples of ARR where senescence is unlikely to be involved or where disease resistance does not increase with the age of plant tissue. Kus *et al.* (2002) showed that ARR of arabidopsis plants to bacterial speck did not require ethylene perception and did not correlate with induction of the senescence marker *SAG-13*. Reuveni *et al.* (1985) showed that younger leaves on mature tobacco plants were more resistant to blue mould than older ones, although the removal of inflorescences could have affected resistance (Fraser, 1981). However, there are more notable exceptions where plant tissues become more susceptible to disease as they age. Older leaves of *Schefflera arboricola* plants, mature wheat plants and onion plants before bulb maturity are more susceptible than younger leaves to *Pseudomonas cichorii* (Chase, 1985), *Puccinia striiformis* (Quayoum and Line, 1985) and *Alternaria porri* (Miller, 1983), respectively. These pathogens presumably have mechanisms to overcome any ARR provided by leaf senescence, if it exists in these hosts.

D. RACE-SPECIFIC SEEDLING RESISTANCE

Race-specific gene-for-gene resistance (GFGR) that protects both seedlings and mature plants is encoded by many well-studied resistance genes, including the *RPP* family of downy mildew (*Peronospora parasitica*) resistance genes from *Arabidopsis* (Holub *et al*., 1994; Reignault *et al*., 1996). The analysis of *Arabidopsis* mutants that affect GFGR and cloning of genes required for resistance has allowed the dissection of signalling pathways involved in GFGR. However, this area has been extensively reviewed (e.g. Dong, 1998; Parker, 2000; Glazebrook, 2001), so is covered only very briefly here. GFGR is thought to be triggered by a direct or indirect interaction between an elicitor produced by the invading pathogen and a resistance protein in the plant. In general, early signalling steps in GFGR are thought to be pathway-specific, but activate more general resistance mechanisms that typically result in the hypersensitive response (HR), a form of programmed death of plant cells that restricts pathogen growth, and the production of effector molecules. Active oxygen species (AOS)

are produced soon after infection during GFGR, and studies in soybean cell suspension cultures suggest that the combined production of nitric oxide and AOS following infection may potentiate the HR (Delledonne *et al.*, 2001). Pathogenesis-related (PR) proteins, defensins, thionins and phytoalexins are the major effector molecules upregulated by various forms of GFGR. These effector molecules are also upregulated during non-specific resistance (Glazebrook *et al.*, 1994; Epple *et al.*, 1997; Vignutelli *et al.*, 1998), although some PR proteins are induced more rapidly (Danhash *et al.*, 1993; Meier *et al.*, 1993; Ashfield *et al.*, 1994) and thionins may be induced to a higher level (Epple *et al.*, 1998) as a result of GFGR. The NPR1 signalling protein is activated by a burst of SA generated during some forms of GFGR and interacts directly with transcription factors that bind to and presumably activate the SA response element in the promoter of PR-1 (Zhou *et al.*, 2000). Transcriptional activation of the defensin gene *PDF2.1* depends on ethylene and jasmonic acid signalling (Epple *et al.*, 1997), the thionin gene (*THI2.1*) is induced by jasmonic acid (Epple *et al.*, 1995) and the upregulation of camalexin probably requires SA (Zhou *et al.*, 1998). However, there is considerable cross-talk between these signalling pathways. For example, jasmonic acid has an antagonistic effect on the defence pathway controlled by SA and vice versa (Gupta *et al.*, 2000; Niki *et al.*, 1998). In addition, SA and ethylene appear to act synergistically to induce PR-1 (Xu *et al.*, 1994). Many components of the GFGR pathways in *Arabidopsis* are shared by other plant defence pathways. For example, jasmonic acid, ethylene and NPR1 are required for ISR (Pieterse *et al.*, 1998). An increase in the production of SA and PR proteins occurs during SAR, which also requires NPR1 (for a review see Barker, 2000). Potentially, this cross-talk could extend to ARR in *Arabidopsis* and other plant species.

The *Cf-2* (Dixon *et al.*, 1996), *Cf-4* (Thomas *et al.*, 1997), *Hcr9-4E* (Takken *et al.*, 1999), *Cf-5* (Dixon *et al.*, 1998) and *Cf-9* (Jones *et al.*, 1994) leaf mould resistance genes from tomato (Hammond-Kosack and Jones, 1993) encode gene-for-gene resistance that protects seedlings and mature plants. In contrast, *Cf-9B* (Panter *et al.*, 2002) and probably *Hcr9-9E* (Parniske *et al.*, 1997) encode race-specific ARR, possibly employing a similar means of pathogen recognition and the same signalling pathways utilised by GFGR in seedlings. The high degree of sequence homology between the C-terminal halves of proteins encoded by the *Cf-9* (Jones *et al.*, 1994), and *Cf-9B* (Parniske *et al.*, 1997) genes suggests that they probably share downstream signalling partners.

V. POSSIBLE MECHANISMS FOR ARR

A. NOVEL DEFENCE PATHWAYS

The characteristics of ARR in different pathosystems suggests that a variety of mechanisms are involved. Some forms of ARR conform to the gene-for-gene model, show similarity to SAR or appear quite distinct from other forms of

resistance. The onset of some forms of ARR correlates with the vegetative–floral transition or, in other cases, the onset of senescence in the oldest leaves of a plant. Based on these obsevations, we propose two additional pathways that may contribute to ARR, namely, senescence-induced resistance (SIR) and flowering-induced resistance (FIR) (Fig. 1).

1. Senescence-induced Resistance

The onset of resistance to blue mould in tobacco plants appears to correlate with the onset of senescence in the oldest leaves rather than the vegetative to floral transition (Wyatt *et al*., 1991; Wyatt and Kuc, 1992). This suggests that senescence of old leaves may generate a signal that induces systemic resistance to blue mould. Other forms of ARR provide resistance that correlates with leaf age in mature plants, suggesting that DR proteins, induced during leaf senescence, could be contributing to resistance. The hypothesis of senescence-induced resistance (SIR) could be analysed further by testing the dependence of some forms of ARR on ethylene perception, SA accumulation, the presence of senescence-specific signalling components, and the induction of DR proteins. Since leaf senescence is not involved in the ARR of *Arabidopsis* to bacterial speck (Kus *et al*., 2002), homologues of *Arabidopsis* genes involved in the induction of DR proteins during senescence, such as *ETR1*, *ORE1*, *ORE9, PAD4*, *NPR1* (Morris *et al*., 2000; reviewed in Quirino *et al*., 2000), and genes encoding the DR proteins themselves, would need to be identified in other species such as tobacco where SIR is suspected. Antisense suppression of these homologues and the comparison of microarray data collected during ARR and senescence could allow the possibility of SIR to be explored in the future.

2. Flowering-induced Resistance

The onset of some other types of ARR seems to correlate with the vegetative–floral transition, rather than the onset of leaf senescence, including resistance of tobacco and *Arabidopsis* plants to black shank disease (Hugot *et al*., 1999) and bacterial speck (Kus *et al*., 2002), respectively. Correlation of SA and PR protein production with flowering in tobacco is additional evidence for the role of flowering in the activation of ARR. Although ARR in *Arabidopsis* was also induced with stress (Kus *et al*., 2002), the authors noted that stress has been implicated in premature flowering, which could presumably trigger ARR. ARR in *Arabidopsis* is dependent on SA, but otherwise appears to involve a novel pathway that leads to the induced production of heat-resistant antibiotic molecules. Identification of other components of this form of ARR in *Arabidopsis* should allow further testing of hypotheses about flowering-induced resistance (FIR). The potential cross-talk between SIR, SAR and FIR is worth investigating because of a common requirement for SA (Quirino *et al*., 1999; Morris *et al*., 2000). Flowers are thought to have evolved as modified leaves, and flower petals undergo senescence (Rubenstein *et al*., 2000). Hence, some

examples of FIR could actually be the result of SIR caused by flower senescence.

B. MULTIPLE RESISTANCE PATHWAYS MAY CONTRIBUTE TO ARR

ARR could be the net result of contributions from different resistance pathways. A durable form of resistance in cereals that has been described variously as 'general', 'partial' or 'horizontal' (reviewed in Loegering, 1984; Hulbert, 1997; Dorrance and Schmitthenner, 2000) is thought to be the result of multiple genes, each of small effect, contributing quantitatively to non-specific resistance. General resistance in maize provides non-specific mature plant resistance to common rust (reviewed in Hulbert, 1997). The durability of this resistance may be due to natural 'pyramiding' of weak resistance genes, but this may be an oversimplification. The contribution of multiple signalling pathways to resistance, including ARR, could occur more widely than previously thought.

1. ARR to Tobacco Black Shank Disease

ARR of tobacco plants to black shank disease (Hugot *et al.*, 1999) may involve signalling pathways that provide FIR, SIR and SAR. One component of ARR is SA-dependent, linking it to SAR and hypothetical SA-dependent SIR and FIR pathways, although the nature of the antibiotic effector molecules is unknown. Another component of ARR is SA-independent, suggesting that it could be the result of flux through the ISR pathway or hypothetical SA-independent SIR or FIR pathways. Identification of the antibiotic compounds induced during ARR and their comparison to known markers for SAR and senesence, and analysis of the ethylene or jasmonic acid dependence of ARR is necessary to distinguish between these possibilities.

2. ARR to Rice Bacterial Blight and Tomato Leaf Mould

Weak ARR to rice bacterial blight and tomato leaf mould has been observed in genetically susceptible plants (Mazzola *et al.*, 1994; S. N. Panter, unpublished data). This raises the possibility that resistance mediated by *Cf-9B* and *Xa21* may be provided by a combination of constitutive, specific and age-related, non-specific components. The race-specific component could be too weak to limit infection in seedlings, but the sum of both forms of resistance could protect mature plants effectively. Isolating and studying the effects of mutants in the age-related non-specific resistance of rice and tomato on *Xa21* and *Cf-9B*-mediated resistance is a possible means of testing this model. The *ISR1* gene, which controls basal resistance and ISR in *Arabidopsis*, is not required for ARR to *P. syringae*. However, in the light of differences between the various examples of ARR, it is conceivable that non-specific resistance controlled by *ISR1* homologues could nevertheless contribute towards *Xa21* and *Cf-9B*-mediated resistance.

VI. MODEL SYSTEMS FOR FURTHER CHARACTERISATION OF ARR

A wide range of resistance phenomena have been described as ‘age-related’, including plant defences that are race-specific or non-specific, SA-dependent or SA-independent, dependent on preformed defences or dependent on inducible defences. While some forms of ARR show similarity to well-characterised plant defences, others do not. Furthermore, most forms of ARR in crop plants have not been characterised beyond their identification as heritable traits of interest to plant breeders. This section discusses the relative advantages and disadvantages of three pathosystems for future molecular studies of ARR.

A. *ARABIDOPSIS*–BACTERIAL SPECK

Arabidopsis thaliana has numerous advantages for plant molecular biology, including a short generation time, the small size of individual plants, a relatively small genome size, the availability of complete genome sequence information and T-DNA or transposon-tagged mutant plants, and well-established protocols for plant growth and pathogen inoculation. There are many *Arabidopsis* mutants that affect plant defence (reviewed in Glazebrook, 2001). Marker proteins for SAR (Uknes *et al*., 1992) and leaf senescence (for a review Quirino *et al*., 2000) have also been identified. These advantages have allowed ISR, SAR, and senescence to be excluded as possible pathways mediating ARR against bacterial speck in *Arabidopsis*, which has instead been linked to flowering and stress (Kus *et al*., 2002). The spectrum of protection provided by ARR, the distribution of ARR among *Arabidopsis* ecotypes and the heritability of this type of ARR needs to be tested further. Despite the advantages of *Arabidopsis*, the usefulness of the *Arabidopsis*–bacterial speck pathosystem is limited, since most pathogens of major crops are fungi. Screens for mutants in ARR, the analysis of ARR in additional known mutants, the identification of proteins and antibiotic molecules produced at the onset of ARR and the testing of its effectiveness against oomycete or fungal pathogens are possible steps in the further characterisation of this novel pathway. The timing of the onset of ARR in Col-0 plants grown under long-daylength conditions and in vernalisation mutants with delayed flowering (reviewed in Pineiro and Coupland, 1998) could provide evidence in support of FIR. Abscisic acid signalling (for a review see Giraudat, 1995) and MAP kinase activity (Jonak *et al*., 1996) are both induced by drought stress, and their involvement in ARR could be tested by pathogen assays of plants with the *abi1* mutation defective in abscisic acid production or perception (Giraudat *et al*., 1994) and plants with altered expression of drought-inducible map kinases (Jonak *et al*., 1996). RNA from inoculated arabidopsis seedlings and mature plants could be used to probe microarrays with defence-induced genes in order to generate a ‘fingerprint’ of genes transcriptionally activated by ARR, which could be compared to the ranges of genes induced by other defence pathways.

B. TOBACCO-BLACK SHANK AND BLUE MOULD

Tobacco is unusual in that it exhibits non-specific ARR to two oomycete pathogens. However, the same broad-spectrum resistance mechanisms could be involved in both cases, by analogy to ARR in *Arabidopsis*, which is effective against two different *Pseudomonas syringae* pathovars. The onset of ARR in tobacco correlates with the senescence of older leaves and/or the vegetative–floral transition. The production of SA and PR proteins at the onset of ARR suggests that the SAR pathway may be involved. Further physiological and biochemical studies that focus on SA and ethylene signalling could look for additional similarities between ARR and SAR and allow the possibilities of FIR and SIR to be explored (see Fig. 1). The SA-independent pathway for ARR to blue mould could be explored further. However, tobacco is a tetraploid plant and does not have as many advantages as the *Arabidopsis* system for rapidly identifying genes that control non-specific ARR. Screening for similar ARR mechanisms in diploid relatives of tobacco or in other solanaceous crop plants is a possible step in the further characterisation of this pathway.

C. TOMATO-LEAF MOULD

The tomato-leaf mould pathosystem has advantages for molecular studies of race-specific ARR that would complement the study of non-specific ARR in *Arabidopsis* and tobacco. These are: a family of resistance genes with members that provide either mature plant or seedling resistance (Jones *et al.*, 1994; Dixon *et al.*, 1996; Parniske *et al.*, 1997; Thomas *et al.*, 1997; Dixon *et al.*, 1998; Takken *et al.*, 1999; Panter *et al.*, 2002), current efforts to identify signalling proteins in *Cf-9*-mediated resistance (Rivas *et al.*, 2002), the availability of methods for purifying specific elicitors of *C. fulvum* (Scholtens-Toma and De Wit, 1988; Joosten *et al.*, 1994), and the establishment of transient expression systems in tobacco for rapid testing of specificity (Thomas *et al.*, 1999; Van der Hoorn *et al.*, 1999). The *Cf-9* and *Cf-4* genes provide seedling resistance (Hammond-Kosack and Jones, 1993) and the *Cf-9B* (Panter *et al.*, 2002) and *Hcr9-9E* (Parniske *et al.*, 1997) genes provide mature-plant resistance and reduced susceptibility, respectively. Alignment of the amino acid sequences suggests that most of the amino acid variation that determines specificity occurs in the N-terminal halves of proteins encoded by the orthologous *Cf-9* and *Cf-4* gene families (Parniske *et al.*, 1997; Thomas *et al.*, 1997). The C-terminal halves of these proteins, which are likely to mediate downstream signalling (Jones and Jones, 1997), are relatively conserved. These proteins provide various degrees of resistance to *C. fulvum* (Hammond-Kosack and Jones, 1993; Takken *et al.*, 1999; Panter *et al.*, 2002). However, biochemical studies suggest that differences in elicitor stability rather than the use of different signal transduction pathways explain why some forms of *Cf* resistance are more effective at inhibiting fungal growth than others (Van den Ackerveken *et al.*, 1992; Van den

Ackerveken *et al.*, 1993; Joosten *et al.*, 1994, 1997; Veervoort *et al.*, 1997). This in turn suggests that Cf proteins providing ARR utilise the same downstream signalling components as those providing seedling resistance, limiting the determinants of the 'mature onset' of this resistance to upstream signalling partners.

Transcriptional control of resistance genes has been disproved as the most likely explanation for the mature onset of resistance (Century *et al.*, 1999; Panter *et al.*, 2002), but other trivial possibilities need to be tested. Cf proteins involved in ARR or their cognate elicitors could be present in mature plants but not in seedlings because of post-transcriptional regulation (Century *et al.*, 1999). Recently, Cf-9 has been isolated as part of a membrane-bound oligomeric complex of approximately 420 kDa (Rivas *et al.*, 2002). Presumably, Cf resistance proteins involved in ARR form similar complexes and it is possible that the subunit composition of these complexes or their stability changes during plant development. Hence, further study of the tomato–*C. fulvum* pathosystem is potentially the fastest approach to discovering the molecular basis for race-specific ARR.

Mature onset of resistance in the tomato–*C. fulvum* pathosystem could perhaps be explained in the context of the guard hypothesis (Dixon *et al.*, 2000; Dangl and Jones, 2001; Nurnberger and Scheel, 2001). This hypothesis proposes that each race-specific elicitor acts as a virulence factor by binding to a specific plant protein, known as a pathogenicity target. The ensuing protein–protein interaction is thought to switch off plant defences or provide the pathogen with nutrients in genetically susceptible plants. According to the guard hypothesis, race-specific resistance may be caused by a resistance gene product 'guarding' a pathogenicity target and triggering plant defence after recognising a complex formed between the pathogenicity target and the elicitor. Three lines of evidence in support of the guard hypothesis are the role of some elicitors in pathogenicity (Rohe *et al.*, 1995; Bai *et al.*, 2000; Chang *et al.*, 2000), the occurrence of some resistance proteins in multiprotein complexes (Zhou *et al.*, 1995, 1997; Bogdanove and Martin, 2000; Cooley *et al.*, 2000; Leister and Katagiri, 2000; Ren *et al.*, 2000, Rivas *et al.*, 2002) and the paucity of examples of direct interaction between resistance proteins and their cognate elicitors (Boyes *et al.*, 1998; Jia *et al.*, 2000; Luderer *et al.*, 2001), in spite of intense research efforts. The Pto–AvrPto interaction has been described as an example of a resistance protein binding directly to an elicitor (Scofield *et al.*, 1996; Tang *et al.*, 1996), but Pto may be the pathogenicity target of AvrPto, and Prf, another receptor-like protein in the signalling pathway (Salmeron *et al.*, 1994, 1996), may serve as the resistance protein (Jones and Jones, 1997).

There is strong circumstantial evidence for the guard hypothesis in the tomato–leaf mould pathosystem. Avr9 binds with high affinity to a protein on the plasma membranes of tomato plants lacking *Cf-9* and other solanaceous plants (Kooman-Gersmann *et al.*, 1996) and four independent binding studies have failed to show a direct interaction between Cf-9 and Avr9 (Luderer *et al.*, 2001).

Epitope-tagged Cf-9 was identified as a subunit of a 420 kDa membrane-bound hetero-oligomeric complex in the microsomal fraction of *Nicotiana benthamiana* leaves where *Cf-9* had been transiently expressed, or *Cf-9* tobacco leaves or *Cf-9* tobacco cell suspension cultures (Rivas *et al.*, 2002). In addition, Cf-9B interacts with a molecule produced in the leaves of *Nicotiana benthamiana* and causes necrosis, which requires a subset of the amino acid residues within Cf-9B required for race-specific resistance to *C. fulvum* (S. N. Panter, unpublished data). This molecule may be the *N. benthamiana* homologue of the pathogenicity target of Avr-9B, but may be sufficiently divergent in sequence that it is recognised by Cf-9B as being in an 'elicitor-activated' state.

If the mature onset of *Cf-9B*-mediated resistance is caused by the expression of Avr9B only in mature plants, this suggests that race-specific ARR has arisen to counter a means of pathogenicity that is a threat only to mature plants (Fig. 2). Alternatively, the pathogenicity target 'guarded' by Cf-9B may only be expressed in mature plants and not in seedlings either because it normally has a role in plant development, or because it controls a signalling pathway, such as plant defence, that functions in mature plants but not in seedlings. Some

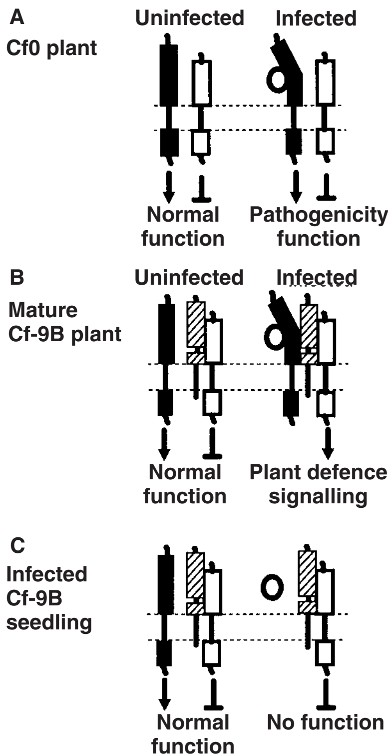

Fig. 2. A model for *Cf-9B*-mediated resistance based on the guard hypothesis. **A.** In Cf0 tomato, the normal function of the pathogenicity target (black) of the Avr9B elicitor (open circle) is modified by an interaction with Avr9B to favour fungal growth *in planta*. Plant defence is not activated because Cf-9B is not present to recognise the elicitor–pathogenicity target complex. **B.** In uninfected mature *Cf-9B* tomato, the pathogenicity target of Avr9B retains its normal function and is not recognised by Cf-9B (striped). During infection with a race of *C. fulvum* expressing Avr9B, the Avr-9B-pathogenicity target complex is recognised by Cf-9B, which activates plant defences via a downstream signalling partner (white). **C.** Tomato seedlings expressing Cf-9B may be susceptible to a race of *C. fulvum* expressing Avr9B due to absence of either Avr9B (left) or its pathogenicity target (right).

non-specific plant defences could have arisen to protect mature plants because they entail a higher 'cost' to seedlings relative to mature plants in terms of diverted resources or tissue damage. Thus, Cf-9B could be guarding a component of a non-specific ARR pathway. If this explanation is true, then Avr9B might be expected to inactivate this underlying resistance pathway in the absence of Cf-9B. A precedent for this model occurs in *Arabidopsis*, where there is strong evidence that the RPM1 seedling resistance protein guards RIN4, a negative regulator of a non-specific resistance pathway, from phosphorylation and activation by the AvrRPM1 and AvrB pathogenicity factors of *P. syringae* (Mackey *et al*., 2002). Another precedent might be Pto, which activates EREBP (ethylene response element binding protein) factors thought to be involved in the transcriptional induction of plant defence genes, if Pto is indeed guarded by Prf (Zhou *et al.*, 1997). The identification of Avr9B and the signalling partners of Cf-9B is likely to distinguish between these possible explanations for the molecular basis of race-specific ARR.

VII. CONCLUDING REMARKS

Some pathogens readily overcome seedling resistance encoded by a single gene. Hence, the identification of additional resistance genes encoding race-specific forms of ARR may be valuable for crop breeding in the future. The detailed characterisation of non-specific forms of ARR in model pathosystems may reveal the extent to which this form of resistance could be manipulated genetically, or by molecular techniques, and used to increase the durability of disease resistance in crops. Studies aiming to find the molecular basis for the mature onset of the various forms of ARR could provide valuable insights into the connections between the various signalling pathways involved in disease resistance and plant development.

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The Origin and Evolution of Tertiary Relict Floras

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ABSTRACT

Tertiary relict floras contain survivors from plant communities that were distributed throughout a large part of the Northern Hemisphere during much of the Tertiary (i.e. 65–15 million years ago (Ma)). They are now mainly restricted to warm humid areas (refugia) in southeastern and western North America, East Asia and southwest Eurasia. Recent molecular phylogenetic studies show that within East Asia the Tertiary relict flora is best divided into two distinct refugial groups, with geographical distributions centred on the Japan/Korea/northeast China and southeast China/Himalayas regions respectively. Recognition of this division leads to a significant improvement in our understanding of the origins and evolution of Tertiary relict floras in East Asia and elsewhere. Molecular studies also indicate two putative clusters of divergence times for East Asian–North American Tertiary relict disjuncts occurring at 5 and 10 Ma. These clusters might reflect a break in the continuity of the Tertiary flora between East Asia and North America across Beringia during a cold period 6–8 Ma, i.e. before the Bering Land Bridge was severed approximately 5 Ma. In addition, there is some evidence that evergreen disjuncts diverged earlier than their deciduous counterparts, possibly due to the high latitude of Beringia. Molecular studies further suggest that divergence times for transatlantic Tertiary relict disjuncts generally fall between 10 and 40 Ma, even though most geological evidence shows that the North Atlantic Land Bridge (NALB), which connected the floras of Europe and North America, was severed around 50 Ma. This raises the issue of whether a partial NALB allowed migration of floras between Eurasia and North America throughout much of the Tertiary. Tertiary relict floras are notable for exhibiting slow morphological evolution (stasis). This might result from large-scale allopatric speciation, together with stabilising selection.

I. INTRODUCTION

The modern distribution of biota throughout the Northern Hemisphere has been greatly affected by the climatic fluctuations and changing geography of the last 65 million years, i.e. the Tertiary and Quaternary periods. As the world cooled over the last 15 million years, members of a large circumboreal plant community adapted to northern latitudes during the early to mid-Tertiary (65–15 million years ago (Ma)) became restricted to limited regions of equable climate (Wolfe, 1975; Tiffney, 1985a; Hoey and Parks, 1991; Xiang *et al.*, 1998b; Wen, 1999), becoming extinct elsewhere. Such areas in East Asia, west and southeast North America, and southwest Eurasia (i.e. southwest Asia and southeast Europe) (Fig. 1) are now refugia for survivors of these floras, and therefore contain a Tertiary relict flora (Tiffney, 1985a,b; Parks and Wendel, 1990; Wen, 1999; Xiang *et al.*, 2000). Recent reviews have summarised information on what is known about Tertiary plant biogeography of the Northern Hemisphere from molecular (Wen, 1999, 2001) and fossil (Manchester, 1999) data, focusing, in particular, on divergence times indicated by molecular data (Xiang *et al.*, 2000; Donoghue *et al.*, 2001), palaeogeographic factors affecting Tertiary plant distributions and migration (Tiffney and Manchester, 2001), new analytical methods for reconstructing plant distributions using the dispersal–vicariance analysis (DIVA) method (Xiang and Soltis, 2001), and the inclusion of fossil taxa into

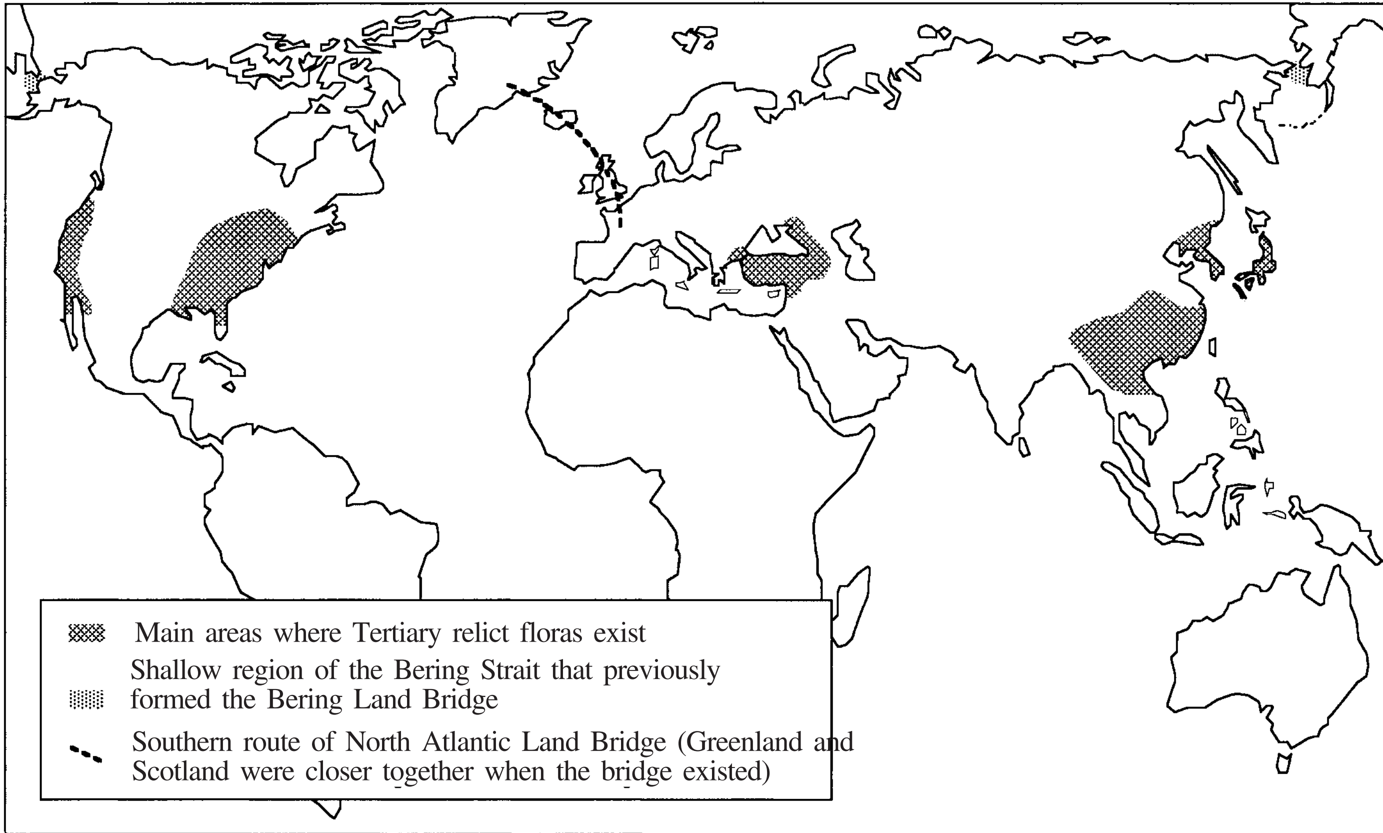

Fig. 1. Principal areas where Tertiary relict floras occur, and probable migration routes between continents.

phylogenies (Manchester and Tiffney, 2001). Here, we concentrate especially on four topics of current interest. First, the existence and causes of a phytogeographic divide within the refugial region of East Asia. Second, the emerging patterns of divergence times among Tertiary relict floras and a putative trend of differing divergence times among evergreen and deciduous taxa. Third, the timing of the severance of the North Atlantic Land Bridge, which acted as a migration route for floras between Europe and North America. Fourth, the cause of slow morphological evolution among Tertiary relict floras, and what might be deduced from this about their histories.

II. TERTIARY RELICT FLORAS

A. ORIGIN

Warm and wet climatic conditions prevailed over northern latitudes, even within the Arctic, for much of the Tertiary and especially during the Eocene (54–34 Ma) and early to mid Miocene (25–10 Ma) periods. During the early Tertiary, plant communities adapted to these conditions were able to move between continents via the Bering Land Bridge (BLB) across the North Pacific, and also via the North Atlantic Land Bridge (NALB). However, migration between Europe and Asia was impeded by an epicontinental seaway, as was migration between east and west North America (Tiffney, 1985a; Tiffney and Manchester, 2001). At the beginning of the Tertiary period, floristic links were in fact stronger across the two land bridges than within continents; hence fossil pollen floras of western North America and Asia were similar (‘*Aquillapollenites*’), whereas eastern North American pollen floras were related to those from Europe (‘*Normapolles*’) (Tiffney, 1985a). As the Tertiary progressed, the seaways bisecting both landmasses receded, and were replaced by regions of dry continental climates, which formed a less effective, but still significant, barrier to migration (Tiffney and Manchester, 2001). Recent work indicates that during the early Tertiary, western North America and eastern Asia were hotspots of evolution and diversification for many Tertiary relict plant genera (and also animals), from which migration then followed to other Northern Hemisphere regions (Xiang and Soltis, 2001). Aided by the land connections and widespread equable climates, a relatively homogeneous flora, that was adapted to warm and wet conditions, was widely distributed throughout a large part of the Northern Hemisphere during the Eocene, and also probably the early Miocene (Tiffney, 1985a,b; Parks and Wendel, 1990; Wen, 1999). During the warm Eocene period this flora was predominantly evergreen and woody, giving way to species-poor deciduous forests only in the extreme north (Tiffney, 1985a, 2000). However, in response to climatic cooling from the start of the Oligocene, deciduous elements moved southwards, and many more deciduous and herbaceous families appeared (Wolfe, 1969, 1978; Tiffney, 1985a), forming a Northern Hemisphere flora (the ‘Mixed Mesophytic Forest’) that comprised a

mix of deciduous and evergreen trees plus increasing numbers of associated herbs (Tiffney, 1985a). Modern Tertiary relict floras comprise mostly deciduous woody taxa, plus some evergreen woody taxa and a small number of herbs.

In response to the late Miocene to Pliocene cooling (5–2 Ma) and Quaternary glaciations (2–0 Ma), this flora retreated to the refugial regions mentioned above (Fig. 1), which retained similar climates (Wolfe, 1975; Tiffney, 1985a,b; Parks and Wendel, 1990; Hoey and Parks, 1991; Wen, 1999; Xiang and Soltis, 2001). This shared history is primarily responsible for the similarity between the modern floras of these regions (Wolfe, 1975; Tiffney, 1985a; Wen, 1999), although in many groups, slow morphological evolution ('stasis') has emphasised this pattern (Wen, 2001). Similar modern distribution patterns among fungi (Wu and Mueller, 1997), freshwater fish (Patterson, 1981) and invertebrates (Suzuki *et al.*, 1977; Enghoff, 1993; Nordlander *et al.*, 1996) indicate that whole biotic communities might have migrated and retreated in the same way as the plant species.

B. FLORISTIC SIMILARITIES AND DIFFERENCES

The remarkable similarity between the floras of parts of North America and East Asia has been known for approximately 150 years (Gray, 1859; Boufford and Spongberg, 1983). Initially, over 100 plant species were considered to be shared between these regions (Boufford and Spongberg, 1983; Wen, 1999). However, subsequent investigation showed that most of these species could be divided into two separate species, i.e. sister species, each being the other's closest relative (Li, 1972; Boufford and Spongberg, 1983; Wen, 1999, 2001; Wen and Shi, 1999). In fact, more recent molecular phylogenetic studies have shown that such species pairs are in most cases not true sister species, because divergence into small clades of species has often occurred within East Asian and/or North American lineages following geographical isolation (vicariance) (Wen and Stuessy, 1993; Wen *et al.*, 1996; Wen, 1999, 2001; Wen and Shi, 1999; Choi and Wen, 2000). Furthermore, many such groups are paraphyletic (Shi *et al.*, 1998; Kim and Kim, 1999; Wen, 1999, 2000, 2001; Hileman *et al.*, 2001; Kim *et al.*, 2001). Molecular data also indicate that many Tertiary relict disjuncts have diverged much less recently than their morphological similarity suggests, indicating unusually slow rates of morphological change (i.e. 'stasis') (Liston *et al.*, 1989a; Parks and Wendel, 1990; Hoey and Parks, 1991; Wen, 1999, 2001).

Species richness among Tertiary relict floras differs greatly between different refugia, with greatest diversity occurring in East Asia, and least diversity in southwest Eurasia (Tiffney, 1985b; Wen, 1999). This reflects different survival and diversification rates within refugial regions (Tiffney, 1985a,b; Wen *et al.*, 1998; Wen, 1999). The survival rate will have depended greatly on how easily plants reached and occupied equable habitats during global cooling and subsequent Quaternary glaciations. In Europe, mountain ranges are thought to have increased extinction rates by blocking southward plant migration (Tiffney,

1985b; Parks and Wendel, 1990; Sang *et al.*, 1997). Conversely, in East Asia, which remained largely free of ice throughout the Quaternary (Hulten, 1937; Frenzel, 1968; Hewitt, 2000), mountains aided survival by interrupting cold northerly air streams (Tiffney, 1985b), without blocking plant migration (Tiffney, 1985a). Consequently, many genera became extinct in Europe, but survived in East Asia following the Miocene (e.g. *Liriodendron*, Parks and Wendel, 1990; *Nyssa*, Wen and Stuessy, 1993; *Aralia*, Wen *et al.*, 1998; *Hamamelis*, Tiffney and Manchester, 2001; see also Sang *et al.*, 1997; Xiang *et al.*, 2000). Diversification within refugial regions would have depended largely on habitat diversity, which is believed to have been greatest in East Asia, and poorer in America and especially southwest Eurasia (Tiffney, 1985a,b; Wen, 1999; Qian and Ricklefs, 2000).

III. PHYTOGEOGRAPHIC DIVIDES WITHIN REFUGIA

A. NORTH AMERICA AND SOUTHWEST EURASIA

Of the three principal areas where Tertiary relict floras occur, i.e. East Asia, southwest Eurasia and North America, only the last is usually considered to be subdivided. North America contains separate refugial areas in the southeast and west (Tiffney, 1985b; Wen *et al.*, 1996; Xiang *et al.*, 1998b; Wen, 1999), separated by the arid centre of the continent which had replaced the early Tertiary epicontinental seaway as a phytogeographic barrier by the middle Tertiary (Tiffney, 1985b; Tiffney and Manchester, 2001). However, the floras of eastern and western North America are generally more closely related to one another than either is to the floras elsewhere (Wen *et al.*, 1996; Xiang *et al.*, 1998b; Wen, 1999; Xiang and Soltis, 2001). Examples of the west North American and east North American members of a Tertiary relict group being derived from separate phylogenetic lineages are very rare (an exception is *Styrax*: Fritsch, 1999, 2001; plate 6a). The phytogeographic barrier provided by the arid centre of North America increased in severity as the Tertiary progressed (Tiffney, 1985b; Tiffney and Manchester, 2001), restricting passage between east and west North America except for taxa tolerant of the developing seasonal and continental climate (Tiffney and Manchester, 2001). However, this barrier might have been breached by less dry-adapted taxa (Wen *et al.*, 1998), perhaps via forest corridors (Tiffney, 1985b). Therefore, the Tertiary refugial regions of west and east North America might for most groups equally be considered subdivisions of a single fragmented massive refugium. Recently, it has been suggested that a further subdivision might exist within eastern North America: between higher and lower altitude Tertiary relict floras, which may have arrived at different times (Donoghue *et al.*, 2001).

Southwest Eurasia has occasionally been cited as two separate floristic regions: southeast Europe and west or southwest Asia (Tiffney, 1985a; Xiang *et al.*, 1998b), but this appears to be a nominal division, and there is no evidence

for a phytogeographic subdivision among Tertiary relicts from these regions. Any apparent subdivision is between floras of different humidity conditions.

B. EAST ASIA

1. A Divide Between the 'Japan' and 'China' Regions

Very recently some workers have begun to recognise that East Asia might be better subdivided into two distinct refugial provinces (Donoghue *et al.*, 2001; Manos and Stanford, 2001; Xiang and Soltis, 2001). The first province is Japan plus Korea, including adjacent areas of northeast China (Manchuria); the second is south and southeast China, with extensions to the Himalayas (Fig. 2). For convenience, we refer to these provinces as 'Japan' and 'China' from here onwards. Among Tertiary relict floras, species from one or other of these two East Asian regions are frequently found to be more closely related to species from America and/or southwest Asia than they are to species from the other East Asian region.

The Japan and China regions are less than 1000 km apart (Fig. 2) and there is no physical (mountain or ocean) barrier to plant migration between the two regions. However, a climatic barrier existed during the Eocene, in the form of an east–west-oriented belt of dry climate separating moister areas in northeast and southeast Asia (Tiffney and Manchester, 2001). This barrier (henceforth, the 'aridity barrier') decreased in intensity towards the Miocene (Tiffney and Manchester, 2001), and even for taxa intolerant of such conditions the divide might have been crossed occasionally by long-distance dispersal or by hypothetical migration corridors. Even now the Japan and China refugial regions are separated by an area of relatively low rainfall (<500 mm, compared to >1000 mm in the refugial regions) stretching west from the mouth of the Hwang Ho river (Bartholomew, 1958; Fig. 2). Conversely, the low rainfall north of 60°N, i.e. around Beringia, might be a modern phenomenon associated with low global temperatures. Although less is known about moisture regimes than temperature in Tertiary Beringian palaeoclimates (Wolfe 1978, 1994), moisture is presumed not to have been a limiting factor to Tertiary relict floras in Beringia (Tiffney and Manchester, 2001).

The Japan–China divide may correspond with a floristic division among the whole East Asian flora, which includes distinct Sino-Himalayan and Sino-Japanese provinces (Wu, 1998). Some genera and species are shared between these two regions; however, others exhibit a clear divide, being present in one region only.

2. Molecular Phylogenetic Evidence

Among Tertiary relict plants, a frequent pattern is for Japanese species to be more closely related to American species than to Chinese ones. In *Corylus*, members of subsection *Siphonochlamys* form a clade that appears to have reached the Japan region recently via the BLB (~3.5 Ma, from molecular data, Whitcher and Wen, 2001, although, from the lifespan of the BLB, 5 Ma is more

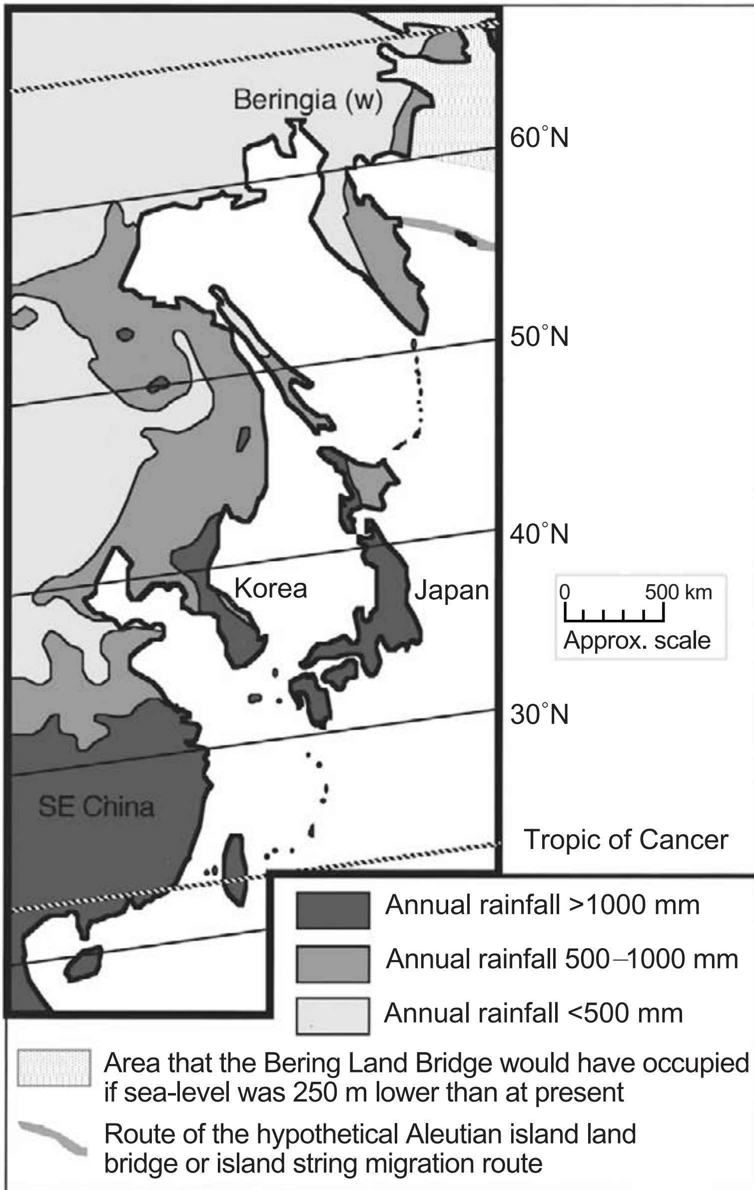

Fig. 2. Annual rainfall in eastern Asia. Areas indicating >1000 mm annual rainfall also correspond approximately with those areas where Tertiary relict floras occur (see Plate 7). The area of >500 mm rainfall at about 38°N corresponds with the ‘aridity barrier’ described in the text. Rainfall data taken from Bartholomew (1958).

likely). Conversely, other East Asian *Corylus* species belong to other subsections, which occur in the China region, and were present >6.5 Ma (Whitcher and Wen, 2001). The single American species of *Hamamelis* is sister to a Japanese species, with the Chinese species sister to this pair (Plate 6b; Wen and Shi, 1999). Similarly, *Panax quinquefolius* (America) forms a clade with *P. japonicus* and *P. ginseng* (Japan), that is nested within a clade of Chinese species (the other American *Panax* species has no close relatives) (Choi and Wen, 2000). In two further cases, Japanese species form clades with American and southwest Eurasian species, but not Chinese species. *Aesculus* (Hippocastanaceae) comprises one clade of only Chinese species, and another with a classic Tertiary relict distribution comprising many American species and one each from southwest Asia and Japan (Xiang *et al.*, 1998a; Plate 6c). *Rhododendron* subgenus *Hymenanthes* comprises two clades, one of which has over 200 species and includes all Himalayan and southeast Chinese species. Of the three Japanese species in the subgenus, one is basal to the above clade. The second clade contains the other two Japanese species, plus three each from North America and southwest Eurasia, but no Chinese species (R. I. Milne, unpublished data).

Several groups exhibit the reverse pattern, i.e. Chinese species are more closely related to American and/or southwest Eurasian species than to Japanese ones. *Aralia* section *Aralia* comprises a clade of American, Himalayan and Chinese species, to which a Japanese species is sister (Wen *et al.*, 1998). *Styrax* contains two species shared between China and eastern North America, one of which is sister to a clade of two Japanese species (Fritsch, 1999; Plate 6a). The Japanese species of *Triosteum* is sister to the clade comprising a clade of two American species and a clade of three Chinese species (Gould and Donoghue, 2000; Donoghue *et al.*, 2001). The single Japanese species of *Castanea* is also basal within the genus, and sister to a clade of Chinese, American and southwest Eurasian species (Manos and Stanford, 2001).

Asarum contains a clade of Japanese species that is sister to two clades of eastern North American species (Kelly, 1998). However, it also contains a second clade of Chinese species from which some American and Eurasian species are derived; hence it exhibits both of the above patterns. However, the Japanese clade has subsequently contributed one species to China, and vice versa (Plate 6d).

In many of the above examples, e.g. *Hamamelis* (Plate 6b), the Japanese and Chinese taxa taken together form a clade that is paraphyletic with respect to a single lineage comprising American and/or southwest Eurasian taxa. In such cases a pattern of dispersal from one East Asian region to the other, followed by dispersal out of the region, could explain the pattern, and if true would indicate that the group probably initially evolved and diverged in East Asia. For these, the aridity barrier could have been involved in the first vicariance event within the group, following which the northern (Japanese) lineage spread eastwards over the BLB (Donoghue *et al.*, 2001; and see Plate 7). Alternatively, the southern

(Chinese) lineage could have spread to Europe, and possibly to America via the NALB. However, the same pattern could equally indicate, for example, dispersal from China to America, followed by dispersal from America to Japan.

There are some cases wherein the China and Japan regions appear to have been colonised by quite separate lineages – most notably in *Aesculus* (Plate 6c) and *Asarum* (Plate 6d), but also in *Corylus* and *Rhododendron*. In groups that arose outside East Asia, the aridity barrier might have kept separate lineages that migrated into East Asia via the BLB, and those that came in via south Eurasia. In addition, some groups might have arisen in East Asia, and given rise to an American or circumboreal clade, which eventually gave rise to a lineage in one East Asian region (for example, this might account for the phylogenetic position of the Japanese species of *Aesculus*, Plate 6c, Xiang *et al.*, 1998a).

Some groups only have a clade in one of the two regions, indicating that, for whatever reason, they either failed to colonise the other region during the Tertiary period, or have become extinct in that region (e.g. *Liquidambar* in Japan; Hoey and Parks, 1991). In many such cases, however, the clade in one region has contributed a species to the other. Both *Eupatorium* (Ito *et al.*, 2000) and *Weigela* (Kim and Kim, 1999; Plate 6e), contain only a single Chinese species, which was derived from within an otherwise Japanese clade. Conversely, in *Torreya* (Donoghue *et al.*, 2001), and *Juglans* (Stanford *et al.*, 2000), the single Japanese species is derived from an otherwise Chinese clade. Clades of *Asarum* in each of China and Japan have contributed single species to the other region. Two *Gleditsea* species from a clade of otherwise American, southwest Asian and Japanese species occur in the China region, which is otherwise occupied by a separate clade of Chinese plus one South American species (Schnabel and Wendel, 1998). The pattern of occasional movement of individual species between the two regions is possibly due to the receding intensity of the aridity barrier between Japan and China during the later Tertiary and into the Quaternary (Tiffney and Manchester, 2001). Migration via the Ryukyu Archipelago and Taiwan is also not impossible in genera capable of short inter-island dispersal (see Plate 7). That a single species is involved in most of these cases indicates that the migration was probably too recent for diversification to have occurred since it happened.

Not all groups with species in both regions exhibit the divide. There is no clear Japan–China divide within *Magnolia* (Kim *et al.*, 2001), *Acer* (Hasebe *et al.*, 1998), *Fagus* or *Quercus* (Manos and Stanford, 2001). In these cases dispersal across the barrier has evidently not been problematic, perhaps because the taxa involved were more drought tolerant than typical Tertiary relict taxa.

3. Pacific and Atlantic Track Relationships

Sister relationships between clades or species in America and those in either Japan or China correspond with a ‘Pacific track’ pattern, which has been defined by Donoghue *et al.* (2001) as one involving a group that (1) comprises representatives in China, Japan, North America but not Europe, and (2) contains trans-

Beringian disjuncts. Donoghue *et al.* (2001) distinguished between Japan and China in defining a complementary pattern, the 'Atlantic track', which applies to groups that (1) have representatives in America, west Eurasia and China but not Japan, and (2) contain transatlantic disjuncts. A problem with these definitions is that absence from southwest Eurasia or Japan might be due to post-Miocene extinction from the region, rather than the lineage not having passed through or existed there. Extinction rates in southwest Eurasia were the highest of any Tertiary relict refugium (see Section II.B), and also were probably higher in Japan than China, due to colder conditions in Japan during Quaternary glaciations (Harrison *et al.*, 2001). One of the mentioned Atlantic track groups, *Liquidambar*, in fact was present in Japan up until the Pliocene (Hoey and Parks, 1991).

Nonetheless, examples of the Pacific and Atlantic track also exist as clades within larger Tertiary relict groups, in cases where the whole group includes extant species in both Europe and Japan, making it less likely that members of the relevant clade became extinct from those regions. Both *Asarum* (Kelly, 1998; Plate 6d) and *Corylus* (respectively subsections *Corylus* and *Siphonochlamys*; Whitcher and Wen, 2001) contain one clade each that exhibits the Atlantic and the Pacific track patterns. *Castanea* provides an especially good example of an Atlantic track pattern, with a clade of southwest Eurasian, American and Chinese species to which the Japanese species is sister (Manos and Stanford, 2001). *Hamamelis* and *Panax* (see Section III.B.2) provide further examples of the Pacific track pattern. Hence, the existence and identification of groups that are distinguished by either a Pacific or Atlantic track pattern of relationship, although complicated by differential extinction rates, appears genuine. Despite this, the subdivision of Pacific track groups into Japan–North America and China–North America vicariants could be more informative.

Japan–North America vicariance is readily explained by relatively recent (i.e. probably Miocene) migration across the BLB (Plate 7). However, sister relationships between China and America which exclude the Japanese lineage are less readily explained. One possible explanation for this is the Japanese lineage having been confined to Japan or thereabouts, while the other lineage migrated around it (Plate 7; Donoghue *et al.*, 2001). This, however, would mean that the migrating lineage must have passed through the aridity barrier to achieve this (Plate 7), which raises the problem of what prevented it from reaching Korea and thence Japan. A scenario which avoids this problem is an initial speciation event within East Asia, leaving one species in the vicinity of Japan and another which diverged once more, providing one lineage that migrated to Beringia, and another that crossed the aridity barrier to reach southeast China.

Other possible explanations for apparent America–China sister relationships include recent extinction from Japan (as occurred in *Liquidambar*) or migration by a route other than the BLB. Some such instances (especially where the American species are all in eastern North America) might in fact represent 'Atlantic track' clades where migration was actually via Europe and the NALB,

followed by extinction in Europe, leaving an apparent America–China sister relationship. This latter hypothesis can be rejected if good fossil data indicate that the lineage was never present in Europe, or if divergence times are too low for migration via the NALB. Divergence times of approximately 10 Ma for *Buckleya* and *Triosteum* (Donoghue *et al.*, 2001) exclude migration via the NALB. However, divergence 13.7–19.7 Ma for the basal split in *Torreya* (Donoghue *et al.*, 2001) does not wholly exclude the NALB, because divergence estimates in this range exist for transatlantic disjuncts (see section VI.B). Indeed, as the accuracy of these increases, divergence time estimates may become the most reliable method of distinguishing between migration via the BLB and via the NALB (Donoghue *et al.*, 2001).

Overall, therefore, the Japan–China divide might in some cases reflect the effects of an initial divide within the region, followed by eastward (Japan to BLB) and westward (China to Europe) migration. Hypotheses that involve direct migration from China to Beringia, bypassing Japan, should be treated with caution. In genera that did not originate in East Asia, it might reflect the region being reached by different routes (probably China from the west, Japan from the east) and/or at different times. A third possibility is origination of a lineage in one East Asian region, from which dispersal to western Eurasia and/or America occurred, followed by dispersal into the other East Asian region. The treatment of Japan and China as separate but adjacent refugial regions clearly allows new perspectives on the biogeography of Tertiary relict floras, and the occasional instance of recent migration of a Tertiary relict group's species between the two should not obscure the pattern.

IV. DISJUNCTION OF TERTIARY RELICT TAXA BETWEEN EAST ASIA AND AMERICA

A. VARIATION IN TIMES OF DISJUNCTION

Although the biogeographic histories of most Tertiary relict groups are likely to be complex (Xiang and Soltis, 2001), the time of divergence between extant Eurasian and American lineages has attracted considerable attention for several reasons. First, it represents a significant event in the lifetime of any group; second, such information can help to pinpoint the timing of important climatic or geological events affecting Tertiary relict floras; and third, vicariance events, particularly recent ones, are at present the only ones whose age may be estimated using molecular data. The timing of a lineage's spread to a new region is equally interesting, but cannot be pinpointed by molecular data, only by a substantial fossil record. It is also generally very difficult to determine the cause of a single vicariance event in any one genus, because of the wide range of factors – biological, climatic and geological – that might have been involved. However, events that affected whole communities can be inferred where many similarly distributed lineages diverged at around the same time.

Among 11 East Asian–North American disjuncts examined by Xiang *et al.* (2000), nine have divergence estimates clustering around 4–5 Ma ago (Plate 8), as do *Symplocarpus* (Wen *et al.*, 1996), *Gleditsea* (Schnabel and Wendel, 1998), *Weigela/Diervilla* (Donoghue *et al.*, 2001), *Magnolia* sect. *Rytidospermum* (Qiu *et al.*, 1995b), and *Calycanthus* and *Boykinia* (Xiang *et al.*, 1998b) (Plate 8). These divergence times correspond well with the opening of the Bering Strait, indicating that this event might have created a barrier to migration between the floras of East Asia and North America and, therefore, led to divergence within many plant groups.

However, among five disjuncts examined and reviewed by Donoghue *et al.*, (2001), a different trend was encountered: there was greater variance in divergence times between genera, ranging from 5 to 18.8 Ma, with only *Weigela/Diervilla* having a divergence time around 5 Ma, whereas all others exceeded 8 Ma. Hence, divergence times among these taxa clustered around 10 Ma. Divergence estimates in this range have also been obtained for East Asian–North American disjuncts in two *Acer* sections (5–11 and 9–15 Ma, based on cpDNA RFLPs; Hasebe *et al.*, 1988; Plate 8), and *Fagus* (10–11.3 Ma, based on the Internal Transcribed Spacers (ITS) of nuclear ribosomal DNA and also noncoding cpDNA sequences; Manos and Stanford, 2001). The only taxon of those examined by Xiang *et al.* (2000) for which the divergence time fell within this time range was the evergreen *Pachysandra*. Relatively early divergence times have also been reported in *Eupatorium/Uncasia* (Ito *et al.*, 2000) and *Paeonia* (Sang *et al.*, 1997). However, in both of these cases divergence times were recorded between two moderately sized clades, indicating that speciation occurred in both groups after the intercontinental disjunction had been achieved. Therefore interspecific breeding barriers might have blocked genetic exchange between continents even if the populations of the genus, taken as a whole, were continuous across a land bridge. In such instances, divergence might not have been correlated with land bridge severing. Taking all molecular data together, it appears that divergence in different groups occurred occasionally throughout the later Tertiary, with a possible peak in divergences around 10 Ma, followed by a second, more obvious, peak at ~5 Ma.

B. CAUSES OF VARIATION

As the Tertiary period progressed, the general trend was of decreasing biogeographic exchange between America and Eurasia. During the Eocene, the two continents were connected by the NALB and BLB, both of which enjoyed warm climates, and the latitude of the NALB was such that light and temperature conditions allowed tropical biota to exist. As the Tertiary progressed, however, the NALB was broken and there was a general cooling trend, reducing the range of taxa to which the BLB was available. Finally, the BLB itself was broken at 5.5–4.8 Ma (Marincovitch and Gladenov, 1999). In addition to severing the land connection, this event also triggered local climatic cooling caused by the mixing

of Arctic and Pacific waters (Sher, 1999), following which, Tertiary relict taxa were probably never again able to occupy this region (Wolfe, 1994). Thus, although a fall in sea-level caused the BLB to reappear during Quaternary glacial epochs (Elias *et al.*, 1996), it would only have harboured genera of arctic or boreal affinity at these times (Hulten, 1937; Murray, 1981). Other than these, only species with small wind-dispersed seeds, or those that could attach to birds (Liston *et al.*, 1989b; Liston and Kadereit, 1995), could have crossed oceanic barriers between America and Eurasia after 5 Ma. The severing of the BLB at 4.8–5.5 Ma therefore provides a likely explanation for the clusters of divergence times of around 5 Ma for Tertiary relict disjuncts.

Other evidence, however, indicates that Tertiary relict taxa might have disappeared from Beringia earlier than this. Palaeoclimate data for the later Miocene, together with fossil plant records from the Bering region itself, indicate that in this vicinity plant communities had become cool- to cold-temperate in nature by between 8 and 5 Ma (Tiffney and Manchester, 2001). Global cooling occurred progressively from approximately 15 Ma (Wolfe, 1978; White *et al.*, 1997; Alroy *et al.*, 2000; Utescher *et al.*, 2000; Plate 8), and some or all Tertiary relict taxa might have disappeared from Beringia by about 8 Ma (Wolfe, 1978). Hence, climatic cooling might be responsible for the cluster of divergence times of around 10 Ma, with the genera concerned having retreated southwards in response, losing the connection across the BLB in the process.

Migration of Tertiary relict taxa across the BLB might have been possible, however, at around 5 Ma, if climates briefly recovered at some point between 7 and 3 Ma, as is suggested by some lines of evidence (Barron, 1973; Wolfe, 1978; Zagwijn and Hager, 1987; Utescher *et al.*, 2000; Lear *et al.*, 2000; Zachos *et al.*, 2001; see Plate 8). If this is correct, climate improvement may have permitted some Tertiary relict taxa to reoccupy Beringia, briefly allowing their populations to become intercontinuous once again via the land bridge, shortly before it severed at approximately 5.5–4.8 Ma (Marincovitch and Gladenov, 1999). Hence, a relatively brief period of climatic warmth, shortly before the BLB disappeared, might account for the cluster of divergence times around 5 Ma for certain groups of disjunct taxa. If so, palaeoclimatic and palaeogeographic data would be in agreement with molecular data, in that clusters of divergence times occurred either side of the cold period around 6–8 Ma.

C. ACCURACY OF MOLECULAR ESTIMATES OF DIVERGENCE TIMES

The conclusion that separate clusters of divergence events for Tertiary relict taxa occurred 5 and 10 Ma must be treated with caution, however, because most of the ~5 Ma estimates are based on one gene sequence, i.e. ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcL*). Conversely, most of the ~10 Ma estimates are based on ITS sequences, and the only *rbcL*-based estimate in this range is evergreen (see Section V). Hence, the possibility that different DNA sequences might tend to over- or under-estimate divergence times must be considered.

Different molecular methods do not always provide compatible divergence estimates when applied to the same disjunct pair. For example, ITS and allozyme markers have produced incompatible divergence estimates for *Menispermum* (Lee *et al.*, 1996; Wen, 1999), *Phyrma* (Lee *et al.*, 1996; Wen, 1999), and *Liquidambar* (Hoey and Parks, 1991; Donoghue *et al.*, 2001; Plate 8). Similarly, *rbcL* sequences provided different, and in most cases lower, divergence estimates for several disjunct pairs (most notably *Campsis*) compared with those produced by earlier studies using different molecular markers (Xiang *et al.*, 2000). Moreover, in many cases, divergence times were miscalculated in earlier studies, and overestimated by a factor of two (Xiang *et al.*, 2000). Hence, molecular divergence estimates cannot in all cases be accurate, and some molecular sequences might eventually be proved to provide more reliable estimates than others.

The *rbcL*-based estimates of Xiang *et al.* (2000) were calibrated using a *Cornus* synonymous substitution clock, but not directly against fossil data within individual genera; rate constancy tests and rate smoothing were applied. Conversely, the ITS-based estimates of Donoghue *et al.* (2001) were calibrated by fossil ancestors of each lineage; however, rates of sequence evolution were not compared between taxa, and rate-constancy tests were not applied. The two methodologies, which might reasonably be described as the most accurate yet employed, indicated separate clusters of divergence time, with one (calculated by Donoghue *et al.*, 2001) being twice as old as the other. It is certainly possible that both clusters of divergence times are genuine because, as noted above, this fits the palaeoclimatic data if it is accepted that a brief warm period occurred between 3 and 7 Ma. However, an alternative hypothesis is that divergence times are underestimated using the *rbcL* sequences, and/or are overestimated using ITS sequences, and hence that divergence times for East Asian–North American disjuncts actually cluster around a single period. Although several other ITS-based divergence estimates indicate divergence between 3 and 6 Ma (Lee *et al.*, 1996), these estimates were not calibrated by fossil data, and so are less accurate than those of Donoghue *et al.* (2001). Strong evidence for clusters of divergence times at both 5 and 10 Ma will only be provided when examination of a large set of disjuncts using a single method yields a bimodal distribution of divergence estimates. This could most rapidly be achieved by examining divergence times for a further set of disjuncts, including those examined by Donoghue *et al.* (2001), using *rbcL* sequences.

V. DISJUNCTION OF TERTIARY RELICT EVERGREEN AND DECIDUOUS TAXA

A. DIFFERENCES IN TIME OF DISJUNCTION

A trend is emerging from molecular data that Tertiary relict evergreen disjuncts might generally have diverged earlier than their deciduous counterparts. Among

11 East Asian–eastern North American disjuncts examined by Xiang *et al.* (2000), eight deciduous taxa had divergence times in the range 0.7–9.8 Ma, all of which could have diverged 3–5 Ma (Plate 8). The exception, *Menispermum*, might have diverged more recently following long-distance seed dispersal, although different molecular markers provide highly discordant divergence times for this genus (Lee *et al.*, 1996; Xiang *et al.*, 2000). Conversely, *Pachysandra*, one of two evergreens surveyed by Xiang *et al.* (2000), diverged 8–17 Ma. The other evergreen surveyed, *Mitchellia*, had a divergence estimate of 2.7–6.4 Ma, although an atypical rate of sequence evolution made this estimate doubtful (Xiang *et al.*, 2000). Other taxa for which a divergence estimate has been obtained of 5 Ma (or less) are all deciduous, i.e. *Symplocarpus* (Wen, 1999), *Weigela/Diervilla* (Donoghue *et al.*, 2001), *Gleditsea* (Schnabel and Wendel, 1998), *Magnolia* sect. *Rytidospermum* (Qiu *et al.*, 1995b), *Boykinia* and *Calycanthus* (Wen *et al.*, 1996).

Among five trans-Pacific disjuncts examined or re-examined by Donoghue *et al.* (2001), three deciduous disjuncts had divergence times under 12 Ma, as did *Fagus* (Manos and Stanford, 2000), which was examined using similar methods. The evergreen gymnosperm *Torreya* diverged 13.7–19.7 Ma, whereas the deciduous *Hamamelis* diverged slightly earlier at 15.5–22.5 Ma (Plate 3).

The family Fagaceae contains both evergreen and deciduous genera exhibiting America–Eurasia disjunctions. American and East Asian species of *Quercus*, and of *Fagus*, diverged respectively *c.*17 and 10–11.3 Ma, whereas the evergreen *Trigonobalanus* disjuncts diverged *c.*37 Ma (Plate 8).

Hence, where groups of genera are compared using the same molecular marker, or from within the same family, evergreen disjuncts tend to have diverged earlier than their deciduous counterparts, with *Hamamelis* and *Mitchellia* being the only genera that break this trend. Divergence estimates for two evergreen *Rhododendron* disjuncts, using *matK* sequence data, are broadly compatible with those for *Pachysandra* and *Torreya*. The American *R. maximum* diverged from its two closest relatives, both southwest Asian, between 6 and 20 Ma; whereas a clade of two other American species diverged from a clade of two Japanese and one southwest Asian species between 6 and 17 Ma (R. I. Milne, unpublished data; Plate 8).

B. CAUSES OF DIFFERENT TIMES OF DISJUNCTION

The Beringia region lies at very high latitude; the narrowest point of the Bering Strait is currently at 65°N, although, depending on sea-levels, the bridge periodically might have extended between 1 and 2 degrees further south. Moreover, the whole region has been moving south throughout the Tertiary, and probably was located 10 to 15 degrees further north during the Eocene (McKenna, 1983; Tiffney, 2000; Tiffney and Manchester, 2001). The high latitude of the region might have acted as a filter to the migration of taxa, preventing some species but not others from migrating across the BLB.

During warm epochs, the ability of warm-adapted, broad-leaved evergreen species to survive at high latitudes was possibly limited not by mean annual temperatures, but by winter sunlight (McKenna, 1983; Tiffney, 1985b, 2000; Tiffney and Manchester, 2001). During the mild but lightless winters experienced, evergreen leaves would have respired throughout, wasting energy that deciduous competitors conserved (Read and Francis, 1992; Tiffney, 2000; Tiffney and Manchester, 2001). Deciduous trees and herbs would be relatively unaffected by such conditions, and, in fact, most taxa that occurred at high latitudes in the early Tertiary, including gymnosperms, were deciduous (Tiffney, 2000). Although fossil evidence indicates that some evergreen taxa did occur within the Arctic Circle region of Beringia, principally on the Alaskan side, during the Eocene period, the original latitude of most such fossils is uncertain due to tectonic activity, and these taxa originally might have occurred further south (Tiffney, 2000; Tiffney and Manchester, 2001).

The southward movement of the BLB, combined with a general decrease in temperature, would have reduced the severity of winter sunlight effects on evergreens as the Tertiary progressed. However, increasing seasonality during the middle Tertiary led to the evolution, diversification and spread of large numbers of deciduous taxa (Wolfe, 1969, 1978; Tiffney, 1985a). Therefore, competition at high latitudes from better-adapted deciduous species is likely to have increased in severity as the Tertiary progressed. This might possibly have made the BLB less equable to evergreen survival, rather than more so, in the later relative to the early Tertiary.

Limited winter sunlight combined with mild temperatures possibly prevented evergreen Tertiary relict genera from being present and migrating at high latitude via the BLB, and instead caused them to use another land bridge which severed earlier. This could have been the NALB, or a hypothetical bridge following the Aleutian Island Arc (56–60°N) (Hamilton, 1983; McKenna, 1983; Qiu *et al.*, 1995b; Wen, 1999; Plate 7), both of which would have occupied lower latitudes more equable to evergreen survival. Migration via the NALB would be indicated for evergreen disjuncts if a trend emerged of similar divergence times between these and transatlantic Tertiary relict disjuncts. The Aleutian bridge hypothesis is difficult to evaluate from phylogenetic data as it links the same refugial regions as does the BLB. It would, however, be tentatively supported as one possible explanation, if evergreen disjuncts were shown to cluster at a time earlier than for deciduous disjuncts, but later than for transatlantic disjuncts. A clear understanding of the lifespan of the NALB is therefore highly desirable (see section VI).

Another possibility that should be considered is that the BLB might periodically have been open to migration by taxa not tolerant of very high latitudes, if its southern margin varied in its southward extent. The BLB's southern margin was certainly in flux due to intense tectonic activity during the early Tertiary (Hamilton, 1983; Tiffney, 1985a, 2000; Tiffney and Manchester, 2001), and it is not impossible that some such activity continued into the middle Tertiary. Therefore tectonic activity and/or periods of low sea-level could have allowed

evergeen taxa to exist at, and migrate via, the BLB's southern margin for short periods. At present, there is no hard evidence for or against this possibility.

In the case of Fagaceae (section V.A), evergreen *Trigonobalanus* is subtropical, whereas deciduous *Quercus* and *Fagus* are mainly warm temperate. Migration among subtropical evergreens could have been blocked by barriers of winter climate as well as by winter light, and so the effect of winter light alone might not easily be deduced from divergence times in such taxa. Divergence in tropical taxa might coincide with the breaking of the NALB and/or global cooling around the late Eocene.

VI. LIFESPAN OF THE NORTH ATLANTIC LAND BRIDGE

A. GEOLOGICAL EVIDENCE

The lifespan of the North Atlantic Land Bridge that connected the Tertiary floras of Eurasia and North America remains a topic of active debate (Tiffney, 2000). The most important connection was via southern Greenland and Scotland, and it is generally accepted that the existing landmasses of Greenland and Scotland parted in the early Eocene, around 50 Ma (Tiffney, 1985b, 2000; Tiffney and Manchester, 2001). A second, more northerly connection existed between north Greenland and Fennoscandia (Tiffney, 1985b), but the biogeographic importance of this is less certain. Although this link persisted until ~40 Ma, it was located 10 to 15 degrees further north, and Fennoscandia was separated by seaways from southern Europe, and also Asia, at this time (Tiffney, 1985b). However, the southern land bridge might have persisted, or periodically reappeared, during some parts of the middle Tertiary (Tiffney, 2000), possibly even up until the early Miocene, due to the activity of the Iceland hotspot (McKenna, 1983). If not as a complete land bridge, it might still have existed as a chain of large islands ('stepping stones'), between which gene flow could occur, for some time after the Eocene. Even now, a submerged basaltic ridge that is <500 m deep for almost all of its length connects Scotland and Greenland, and is emergent at both Iceland and the Faröes. If this had been raised 500 m relative to sea-level then a possibly continuous bridge would have resulted; a lesser rise would have produced island stepping stones.

Most of the geophysical evidence available indicates that the land bridge was broken during the Eocene (see Tiffney, 2000, for references), although this precludes neither the subsequent brief reappearance of the bridge nor the continued existence of an island string (Tiffney, 2000). Two studies, however, have indicated a longer lifespan for the NALB (up until 25 or even 15 Ma) based on ocean microfauna (Poole and Vorren, 1993) and ocean circulation patterns (Schnitker, 1980).

The fossil record also does not provide an unequivocal answer. European and American mammal faunas began to diverge after the Eocene, but floras remained very similar up to the Miocene (Manchester, 1999; Tiffney and Manchester,

2001). The floral similarity could reflect divergence in the Eocene followed by morphological stasis (Manchester, 1999; Tiffney, 2000; Tiffney and Manchester, 2001; also see discussion on stasis in section VII). However, it might also indicate that plants remained able to cross the NALB for much longer than did animals, perhaps because they could migrate across gaps in the bridge. The larger the gaps in the bridge, the more important seed dispersability would become for migrating plants (Tiffney, 2000). Any North Atlantic land connection or island string would likely have provided equable climates for Tertiary relict taxa in the early Miocene (Tiffney, 2000), as both *Liriodendron* and *Magnolia* existed in Iceland at this time (McKenna, 1983).

B. DIVERGENCE TIMES OF TRANSATLANTIC DISJUNCTS

Transatlantic plant divergence times provide a strong source of evidence regarding the lifespan of transatlantic land migration routes. As well as taxa that have the humid requirements typical of Tertiary relict groups, transatlantic disjuncts also exist among sclerophyllous floras of drier conditions, as many such genera occur disjunctly in southern Europe (Mediterranean) and southern North America (Thorne, 1972; Axelrod, 1975). Although such floras are likely to have been distributed differently to Tertiary relict floras proper, inhabiting drier regions, throughout the Tertiary (Axelrod, 1975; Fritsch, 1996), some of the genera involved nonetheless exhibit very similar modern distribution patterns to Tertiary relict taxa (e.g. *Cercis*, Hao *et al.*, 2001; Donoghue *et al.*, 2001; *Styrax*, Fritsch, 1996, 1999, 2001). Although some authors dispute this (Axelrod, 1975; Fritsch, 1996, 2001), these taxa might have employed similar migration routes to Tertiary relict floras proper (specifically the NALB), although perhaps during different (drier) epochs, such as the late Oligocene. The greater prevalence of transatlantic disjunctions in floras of drier climates, relative to less dry-adapted Tertiary relict genera, might reflect lower Pliocene/Quaternary extinction rates of European lineages among the former. The two are therefore considered together here.

The importance of the NALB is not restricted to modern southwest Eurasia–North America disjuncts. During the early Eocene it was almost certainly the principal connection for all biota between America and Eurasia, owing to its lower latitude and tropical climate (Tiffney, 1985b; Tiffney and Manchester, 2001). Probably many of the older Tertiary relict genera initially achieved their intercontinental distribution via this route, e.g. *Juglans* (Stanford *et al.*, 2000). The NALB might also have been a crucial part of a significant migration route between Africa and America (Lavin *et al.*, 2000).

Molecular divergence estimates from transatlantic disjuncts concur in indicating that migration occurred more recently than 40 Ma (Table I); that is, after the Eocene, and after Greenland and Scotland parted. Most divergence estimates fall within a range of 10–40 Ma (Table I), with the exception of *Corylus* (>8 Ma; Whitcher and Wen, 2001), indicating a clear trend of earlier divergence than

among trans-Beringia disjuncts. There is considerable disagreement between genera as to when migration occurred (Table I), although there has not yet been a systematic examination of many transatlantic sister pairs, as there has been for trans-Beringian ones (Xiang *et al.*, 2000; Donoghue *et al.* 2001). The disparity in divergence times might reflect gradual sinking of portions of the bridge causing vicariance in different groups according to their ability to migrate across oceanic barriers. However, the two genera with the most recent estimates have seeds that seem maladapted for crossing large ocean gaps between land. *Styrax* seeds are ant-dispersed (Fritsch, 1996), whereas hazelnuts (*Corylus*) are too large to have been carried long distances intact by birds.

C. ALTERNATIVE MIGRATION ROUTES TO THE NORTH ATLANTIC LAND BRIDGE

For taxa that could not tolerate the high latitude of the BLB, the NALB was probably the only migration route between Eurasia and America available at any point during the Tertiary period. For tropical or subtropical evergreens in particular (such as *Trigonobalanus* and associated taxa; Manos and Stanford, 2001), it represents the only likely route of migration between the two landmasses. For evergreen taxa of temperate or warm temperate affinity, it is debatable whether migration via the BLB was ever possible, depending on the effect of winter darkness, coupled with temperature regimes and competition, on such taxa. In cases where such a group is extant in East Asia and North America but not Europe, the NALB should be seriously considered as a migration route, especially for those groups which, according to fossil evidence, formerly occurred in Europe, and/or whose intercontinental divergence is dated as earlier than for most associated deciduous disjuncts. Because phylogenies based on extant species are inevitably incomplete due to extinctions (especially in southwest Eurasia), the presence of extant sister species or clades in East Asia and North America does not necessarily indicate migration via the Bering route. One possible approach to addressing this problem is to compare divergence times among three categories of disjuncts; i.e. transatlantic, evergreen America–Eurasia, and deciduous America–East Asia disjunct pairs, using the same molecular markers in all cases. Similar clusters of divergence times among transatlantic and evergreen disjuncts would indicate migration via the same route, especially if deciduous America–East Asia disjuncts generally diverged later. However, divergence times for transatlantic disjuncts might not cluster around a single period, if the gradual sinking of portions of the NALB cut off populations one by one according to their dispersive capabilities.

Only one route other than the NALB has been postulated for direct migration between southwest Eurasia and North America. That is the 'Madrean–Tethyan' route, wherein a string of volcanic islands at the latitude of the modern Azores allowed migration by 'island-hopping' between the two continents between 25 and 38 Ma (Axelrod, 1975; Liston *et al.*, 1989a). The suggested lifespan of such a connection is similar to that which a post-Eocene NALB would need

TABLE I
Divergence times for transatlantic disjunct pairs

Genus	Divergence time (Ma)	Molecular marker	Climate	Reference
Datisca	10–40[a]	Isozymes and cpDNA[a]	Subhumid	Liston *et al.* (1989a) Liston *et al.* (1992)
Corylus ss. *Corylus*	>8[b]	ITS (internal transcribed spacer) sequence[c]	Subtropical to temperate	Whitcher and Wen (2001)
Styrax	5–13.8	Isozymes	Subhumid	Fritsch (1996, 1999)
Cercis	13.1–17.7 (9–32)	ITS and *ndhF*	Subhumid	Donoghue *et al.* (2001)
Liquidambar	29.5–40.5	ITS	Humid	Hoey and Parks (1991) Li and Donoghue (1999)
Rhododendron ss. *Pontica*	6.5–20	Plastid DNA sequence (*matK*)	Humid	R. I. Milne (unpublished data)

[a] Isozyme data originally indicated a 10–40 Ma range for *Datisca* (Liston *et al.*, 1989a), but Fritsch (1996) recalculated the divergence time and stated that 30–40 Ma was more appropriate. However *rbcL* data indicate divergence later than 30 Ma (Swensen *et al.*, 1994). If the *rbcL* sequence evolved at an approximately constant rate, the divergence of the two *Datisca* species examined is about one-fifth as old as that of Fabales, Rosaceae and Cucurbitales (which include *Datisca*) (Swensen *et al.*, 1994). This split appears to have occurred around 80–95 Ma (Wikstrom *et al.*, 2001) and from this, therefore, the *Datisca* split occurred 16–19 Ma.
[b] Tamura–Nei distance of 0, which indicates recent divergence, but prevents accurate assessment of time since divergence (Whitcher and Wen, 2001).
[c] The transatlantic relationship is not supported by *matK* data (Mehlenbacher and Erdogan, 2001). If neither phylogeny is rendered inaccurate by convergent mutations, reticulate evolution might explain this.

to have had to account for most of the observed divergence times (Table I). Thus, the two hypotheses cannot readily be distinguished using divergence time estimates. There are groups whose phylogeography fits well the Madrean–Tethyan hypothesis, particularly Amaryllidaceae whose American clade is sister to the European clade but, significantly, appears to have radiated out from a point in Central America (Meerow *et al.*, 2000). However, many other groups which appeared initially to fit the hypothesis were subsequently shown not to be monophyletic (Hileman *et al.*, 2000; Fritsch, 1996). Furthermore, geological evidence for the existence of such an island string is lacking so far (Wolfe, 1975; Fritsch, 1996), while temperatures at the latitude of the hypothetical island string might have been too high for at least some of the taxa involved. Also, as mentioned

above, some of the species concerned seem maladapted for inter-island dispersal (Fritsch, 1996; Whitcher and Wen, 2001). A final argument against this hypothesis, provided by morphological stasis, is discussed in the following section.

To summarise, although it is indicated by molecular data that some taxa migrated across the Atlantic between 40 and 15 Ma, this is disputed by paleogeographic data, which indicate that a viable land connection ceased to exist around 50 Ma. Further molecular examinations of divergence times, particularly comparing many disjuncts using a single molecular sequence, are therefore highly desirable.

VII. CAUSES OF MORPHOLOGICAL STASIS

A. GENETIC CONSTRAINTS AND STABILISING SELECTION

Tertiary relict floras are remarkable for the morphological similarity displayed by groups of species growing on separate continents (Wen, 2001). It was this which caused many disjuncts to be originally described as single species distributed in both North America and East Asia. Given that the majority of these taxa appear to have diverged at least 5 Ma (Wen, 1999; Xiang *et al.*, 2000; Donoghue *et al.*, 2001), and in some cases much longer ago, this morphological similarity must reflect slow rates of morphological evolution, i.e. ‘morphological stasis’ (Williamson, 1987; Liston *et al.*, 1989a; Parks and Wendel, 1990; Hoey and Parks, 1991; Wen, 1999, 2000, 2001). Morphological stasis is commonly accompanied by interfertility between disjuncts (Parks and Wendel, 1990; Qiu *et al.*, 1995b; Wen and Jansen, 1995; Wen, 1999); and by very similar climatic tolerance between disjuncts, their living relatives, and (by inference from fossil communities) their ancestors (Tiffney and Manchester, 2001). Hence, morphological characteristics, genetic factors governing compatibility, and climatic tolerance have all changed very little in vicariants subject to stasis, since they diverged.

There are two commonly cited explanations for this pattern; one is internal genetic constraint preventing significant evolutionary change (Williamson, 1987; Wen, 1999), the other is stabilising selection, i.e. removal of phenotypes that deviate from what is typical for the population (Wen, 2001). The former would act regardless of habitat conditions, but the latter requires habitat and climatic conditions to remain stable over long periods, thus removing the impetus for evolutionary change (Williamson, 1987; Liston *et al.*, 1989a; Parks and Wendel, 1990; Hoey and Parks, 1991; Wen, 1999). Of these, climatic stability has the advantage that it provides an explanation for why stasis should affect many similarly distributed but unrelated groups (Wen, 1999).

A second argument against the constraints hypothesis is the high incidence of paraphyly among Tertiary relict genera (at least, as originally circumscribed). Specifically, from within many genera (or sections) which have a Tertiary relict distribution, there has been derived a morphologically (and often ecologically) distinct genus (or section). The phenomenon is well illustrated by *Liquidambar*,

which comprises four morphologically similar species in East Asia, southwest Eurasia and southeast North America, but according to molecular phylogenetic analysis is paraphyletic with respect to two monotypic genera (Shi *et al.*, 1998; Plate 6f; Table II). A more striking example is *Aralia*, which exhibits paraphyly at each of genus, section and series levels (Wen, 2000, 2001; Table II). The genus *Magnolia*, and its section *Rhytidospermum*, might each either be viewed as polyphyletic, or paraphyletic with respect to multiple lineages (Qiu *et al.*, 1995a; Kim *et al.*, 2001; Table II). At least eight other Tertiary relict groups exhibit paraphyly (Table II; Plate 6e), and in some cases the derived lineage is far more speciose and/or morphologically diverse than the progenitor group (Table II).

Paraphyly is to some extent a function of human perception, i.e. the species of a group like *Liquidambar* were perceived as forming a cohesive morphological unit from which the two derived genera were excluded due to their apparent morphological distinctness. However, paraphyletic groups are generally those in which morphological change in most taxa has proceeded slowly, whereas it has occurred at a relatively fast rate in the derived lineage(s). A good example is *Arbutus*, a genus of warm-adapted trees from within which has been derived a clade comprising five genera and over 70 species, including arctic–alpine subshrubs (Hileman *et al.*, 2001; Table II).

Kim and Kim (1999) remarked that paraphyly is contrary to the morphological stasis hypothesis, but in fact it is only contrary to a hypothesis of stasis by constraints. If internal genetic constraints limited morphological change within a group, then it should not be possible for change to accelerate suddenly in one or more lineages of that group. Where stasis is due to stabilising selection, on the other hand, the rate of evolutionary change could be accelerated in any lineage which became subject to different selective pressures (Wen, 2001). Factors which might have triggered the breaking of stasis, or at least the acceleration of morphological change, in lineages derived from paraphyletic Tertiary relict groups include adaptation to warmer climates (e.g. *Liquidambar*, Shi *et al.*, 1998; *Aralia*, Wen, 2000; and see discussion in Wen, 2001), a new pollination syndrome (*Weigela/Diervilla*, Kim and Kim, 1999), adaptation to a cooler understorey niche (*Aralia hispida*, Wen, 2001); and the uplifting of a mountain range, such as the Rockies (*Osmorhiza*, Wen, 2001) or the Himalayas (*Rhododendron*, R. I. Milne, unpublished data, and see Irving and Hebda, 1993).

Given this evidence against the constraints hypothesis, the continuing climatic similarity between regions containing Tertiary relict floras is probably the more important of the two factors overall. However, the two might act together in some cases (Williamson, 1987), and constraints should not be rejected for relatively old lineages that are not paraphyletic (e.g. *Liriodendron*, Parks and Wendel, 1990). Stabilising selection might act not only to prevent the accumulation of new alleles, but also to promote parallel evolution, i.e. identical genetic changes in each vicariant population in response to the same or similar extrinsic factors (Rieseberg and Burke, 2001).

TABLE II

Paraphyletic Tertiary relict groups and their derived taxa

Paraphyletic group			Derived group			
Genus/group	Distribution[a]	No. of spp.	Genus/genera/group(s)	No. of lineages	Distribution[a]	No. of spp.
Aralia	J, SEA, ENA, WNA	~30	*Pentapanax*	1	SEA	15
Aralia section *Dimorphanthus*	J, SEA, ENA	25	*Aralia* section *Hispidae*	1	ENA	1
Aralia series *Dimorphanthus*	J, SEA, ENA	11	series *Chinensis* and *Foliosae*	1	SEA; SEA	6; 8
Arbutus[b]	SWE, CI, WNA	11	*Arctostaphylos sens. lat.*[b]	1	Circumboreal	62; 2; 9; 1; 1
Diphylleia	J, SEA, ENA	3	*Dysosma, Podophyllum*	1	SEA; ENA, SEA	6; 3
Gordonia	SEA, ENA	14	*Schima* and *Franklinia*	1	SEA; ENA	15; 1
Liquidambar[c]	SEA, SWE, ENA	4	*Semiliquidambar*, *Altingia*	1	SEA; SEA	1; 1
Lithocarpus	J, SEA, WNA	300	*Chrysolepis*	1	WNA	2
Magnolia	J, SEA, ENA, CA	141	Rest of subfam. Magnolioideae[d]	4	J, SEA, CA, SA, M	25; 2; 2; 4; 30
Osmorhiza sect. *Osmorhiza*	J, SEA, ENA	12	Other members of *Osmorhiza*	3	WNA, CA, SA	7
Rhododendron subsect. *Pontica*	J, SEA, SWE, ENA, WNA	11	Rest of subgenus *Hymenanthes*[e]	1	SEA	>200
Stewartia	J, SEA, ENA	13	*Hartia*	2	SEA	18
Weigela[c]	J, SEA	12	*Diervilla*	1	ENA	3

[a] J = Japan, Korea, Manchuria; SEA = southeast Asia, mostly southeast China and Himalayas; SWE = southwest Eurasia; CI = Canary Islands and N Africa; ENA = eastern North America; WNA = west North America; CA = Central America and West Indies; SA = South America; M = Malesia (southeast Asian Islands southeast to New Guinea).

[b] Monophyletic lineage comprising *Arctostaphylos, Arctous, Comarostylis, Ornithostaphylos*, and *Xylococcus* (Hileman *et al.*, 2001).

[c] See Plates 6e (*Weigela*) and 6f (*Liquidambar*).

[d] Comprises five genera other than *Magnolia*; of these, *Manglietia, Kmeria* and *Pachylarynx* appear to be monophyletic and each independently derived from within *Magnolia*, whereas *Elmerilla* and *Michelia* together form a fourth clade derived from *Magnolia*, which also contains members of *Magnolia* sect. *Maingola* (Kim *et al.*, 2001).

[e] Comprises 21 other subsections. Previously each were thought to be as distinct from each other as from subsection Pontica.

Sources of data are as follows: *Aralia* – Wen (2000); *Arbutus* – Hileman *et al.* (2001); *Diphylleia* – Kim and Jansen (1998) and Wen (1999); *Gordonia* – Prince and Parks (2000); *Liquidambar* – Shi *et al.* (1998); *Lithocarpus* – Manos and Stanford (2001); *Magnolia* – Kim *et al.* (2001); *Osmorhiza* – Wen (2001); *Rhododendron* – R. I. Milne, unpublished data; *Stewartia* – Prince and Parks (2000); *Weigela* – Kim and Kim (1999).

B. MODE OF SPECIATION

Both the constraints and the stabilising selection hypotheses describe mechanisms that would act over the lifetime of a species, slowing down gradual evolutionary change. However, morphological and genetic change often occurs in rapid bursts ('punctuated equilibrium'), most frequently accompanying speciation (Levin, 2000). Possibly the rate of change since speciation in Tertiary relict floras is not as different from other floras as it appears to be, and the similarity between disjuncts in fact reflects the results of speciation without an accompanying burst of rapid evolutionary change.

Most Tertiary relict intercontinental disjuncts probably speciated in response to the vicariance of very large populations by physical separation, i.e. severing of land bridges and/or contraction to their current ranges (Wen, 1999; Tiffney, 1985b). This corresponds with the large-scale allopatric or 'geographic' mode of speciation (Levin, 2000), a mechanism that may be relatively common among long-lived woody perennials (Rieseberg and Brouillet, 1994), which most Tertiary relict taxa are. When speciation occurs in this manner, it is not accompanied by any initial impetus towards morphological, genetic or ecological change, and as both daughter species retain large populations, new mutations will be fixed only rarely (Mayr, 1982; Levin, 2000) unless strong selection pressure is subsequently applied (Rieseberg and Burke, 2001). The two populations will diverge only gradually, due to occasional fixation of neutral mutations, and also of mutations which confer slight advantages in response to subtle habitat and climate differences between the regions occupied by the two vicariant populations. Parallel responses to selection may further preserve similarity between them (Rieseberg and Burke, 2001). Hence, the result will be two species with small morphological differences relative to the time since separation, and which are often still inter-fertile with one another. This is exactly what is commonly observed among Tertiary relict disjunct species groups.

Conversely, other mechanisms of speciation, i.e. peripatric and founder-effect speciation, can produce a daughter species that is genetically and morphologically separate from the progenitor species within a relatively short time. This occurs when the speciation process involves one or more small populations, peripheral to the continuous range of a species (Mayr, 1970; Levin, 2000). Such populations can rapidly diverge due to founder effects and genetic drift (Mayr, 1982), and/or selection driven by the occupation of novel habitats (Levin, 2000). In addition to rapid morphological change, they then may become ecologically and genetically isolated from the progenitor in the process of speciation. Therefore, disjuncts subject to stasis might be those that underwent large-scale allopatric speciation, whereas other genera in which there is more between-species variation might be those where speciation has proceeded by other means.

In those Tertiary relict genera that are paraphyletic, speciation might have progressed both with and without accompanying rapid change, in different instances. In peripatric/founder effect speciation models, while new mutations

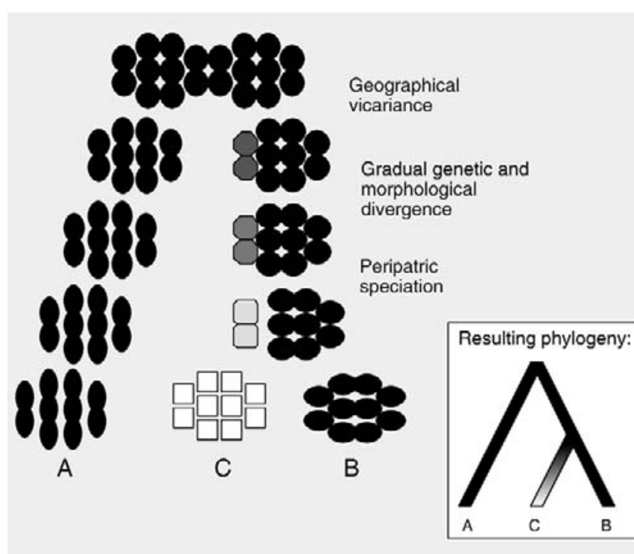

Fig. 3. How large-scale allopatric speciation followed by peripatric speciation might be responsible for the appearance of paraphyly in a disjunct genus.

accumulate rapidly in the small neospecies population(s), the progenitor population remains largely or wholly unchanged (Levin, 2000; see also Rieseberg and Brouillet, 1994). Therefore, if the progenitor has previously undergone geographic speciation, it and its original sister species will resemble each other more closely in morphology than either does the daughter species (Fig. 3). Therefore they will appear to form a natural group that excludes the daughter species, but which in fact is paraphyletic with respect to the daughter species. Variations on this basic pattern might be responsible for some of the combinations of morphological stasis and apparent paraphyly that are commonplace among Tertiary relict groups.

If stasis did result partly from large-scale allopatric speciation, climatic stability must still have played an important role. The apparent retention of climatic tolerances in modern descendants of fossil floras ('Physiological Uniformitarianism', Tiffney and Manchester, 2001) indicates that, had constant climatic conditions not been available, species subject to stasis would have become extinct (as occurred in Europe; Tiffney, 1985a; Parks and Wendel, 1990; Wen and Stuessy, 1993; Wen *et al.*, 1998; Xiang *et al.*, 1998b; Xiang *et al.*, 2000), or evolved into new forms (as appears to have occurred, through hybridisation, in European *Paeonia*; Sang *et al.*, 1997). Most probably, stasis reflects the combined effects of large-scale allopatric speciation followed by stabilising selection over at least most of the lifetimes of the resultant daughter species.

C. STASIS AND MODE OF MIGRATION

If large-scale allopatric speciation was an important factor in creating morphological stasis, then it follows that the populations comprising the two vicariant species that are subject to stasis must previously have been intercontinuous, and therefore connected across a land bridge up until the time that they separated. Hence, species subject to stasis cannot have achieved their disjunct distributions by long-distance dispersal, and especially not by sequential colonisation of a string of oceanic islands. The former would have involved a single founder effect, the latter many. Migration across an oceanic island string would also involve passing through a variety of microhabitats and microclimates, and so is also contrary to a hypothesis of stasis by stabilising selection. This point is illustrated by *Verbascum thapsus*, a biennial herb which was introduced to Hawaii in the early 20th century, and to mainland USA earlier still. Material in Hawaii has undergone substantial changes (woodiness, gigantism and a perennial habit; Juvik and Juvik, 1992) since introduction, whereas during its expansion through the USA, *V. thapsus* has only undergone one notable change: the switch of some southern populations to an annual habit (Reinartz, 1984). Hence, the rate of evolutionary change has been slightly accelerated in mainland America, and greatly accelerated in Hawaii, where the species encountered atypical conditions and adapted to a new niche. The Madrean–Tethyan hypothesis posits sequential founder effects on a number of oceanic islands which were likely tropical in climate. It is highly unlikely that stasis could be maintained through such a process, unless it were due to constraints. However, constraints can be rejected in paraphyletic genera such as *Arbutus* (Hileman *et al.*, 2001), so for such cases the Madrean–Tethyan hypothesis should also be rejected.

The above argument does not apply to island strings that resulted from the sinking of a previously continuous land bridge, because gene flow could be maintained between existing populations on each island, without the occurrence of founder effects. Therefore, stasis by large-scale allopatric speciation is not an argument against gene flow having been maintained for a time across an island chain that was previously the NALB. In the case of the putative Aleutian route between East Asia and Alaska, it is at present unclear whether a land connection ever existed along this route, or if it was always a string of islands (Hamilton, 1983; McKenna, 1983). Hence, it cannot be determined how stasis might have been affected in a lineage that migrated this way.

A single founder effect on a continent, such as would follow long-distance dispersal between continents, would lead to the breaking of stasis only if large population size were important for maintaining stasis. If stabilising selection were the key factor, then the founding individual would likely only survive if it chanced to land in the correct climate zone, and among a community where a niche existed to which it was pre-adapted, in which case stabilising selection might continue to act.

The importance of stabilising selection can be tested indirectly by determining whether different rates of morphological evolution are typical in different biogeographic regions (Wen, 2001). For species with short generation times it might be possible to test the constraints hypothesis by attempting to breed for morphologically divergent forms. However, paraphyly and variable rates of morphological change within genera provide evidence against the constraints hypothesis for stasis, for those taxa where it occurs, and as a general pattern. The increasing scope of molecular markers and population modelling might in future make it possible to determine whether disjunct species have passed through extreme bottlenecks at or since the point when they diverged, and hence test the allopatric speciation hypothesis. Proof of this hypothesis would be a substantial step towards demonstrating that a species migrated over a land bridge between continents, and would be particularly instructive for transatlantic disjuncts indicated to have diverged <40 Ma. A better understanding of the mechanisms underlying stasis would therefore offer new insights into the biogeography of Tertiary relict groups.

VIII. CONCLUSIONS

As molecular phylogenies, divergence times and biogeographic analyses become more accurate and widespread, our understanding of patterns of plant evolution, migration and extinction in the Tertiary is becoming more clear, but remains limited by missing lineages due to extinctions, especially in Europe. In this article, attention has been drawn to several aspects of the historical biogeography and evolution of Tertiary relict floras that have attracted recent interest. The biogeographic divide between China and Japan is a pattern which permits finer analysis of Tertiary relict groups, because the representatives of such groups can now be divided up among five refugial regions rather than four. The divide between these two regions is unusual in that the regions are positioned so close together, and consequently some lineages have probably migrated between the areas with ease, whereas in others (notably *Aesculus*), connection between Japan and China might only have been achieved via at least one other continent. The barrier between the two regions is a band of dry climate that was far more severe in the early Tertiary, although it still exists today.

Divergence times between American and Eurasian disjuncts cluster around 5 and 10 Ma, but it is too early to be certain that both clusters are genuine, because the effects of bias due to different markers and techniques employed must first be eliminated. Ultimately, a situation where all disjuncts are examined and compared using at least one universal method (such as ITS or *rbcL* sequences) is desirable and achievable. Different divergence times might exist between evergreen and deciduous disjuncts. This could be because evergreens were unable to cross the BLB, and hence migration among them ceased when the NALB became unavailable. Most evergreens are tropical, and earlier divergence in such groups

would be expected in any case due to climatic cooling driving them from higher latitudes where intercontinental land bridges existed. However, divergence times among temperate evergreen disjuncts (of which few have so far been examined) would be especially instructive concerning the effects of winter sunlight and whether this prevented them from using the BLB. Systematic comparisons of divergence times among transatlantic and trans-Beringian, evergreen and deciduous disjuncts, are required to address these questions.

Morphological stasis among tertiary relict floras has been attributed to genetic constraints and/or stabilising selection, with more authors preferring the latter. Paraphyly is very common among Tertiary relict floras, and is contrary to the constraints hypothesis. However, a third factor might also have been involved in creating stasis. Large-scale allopatric speciation typically leaves two daughter species with large populations that will remain morphologically similar and inter-fertile for long periods thereafter. Hence, this mechanism should be evaluated as a cause of stasis among Tertiary relict disjuncts, most of which probably speciated in this manner. If this mechanism were proven, it would provide strong evidence of migration via a land connection in Tertiary relict disjuncts that are subject to stasis.

The study of Tertiary relict floras is a highly active research field, where the principal challenge is to work around the incompleteness of both the fossil record and the surviving representatives of formerly widespread lineages. As molecular techniques become more sensitive, additional patterns of diversity, migration and evolution may emerge, leading to further improvement to our understanding of the historical biogeography of Tertiary biota.

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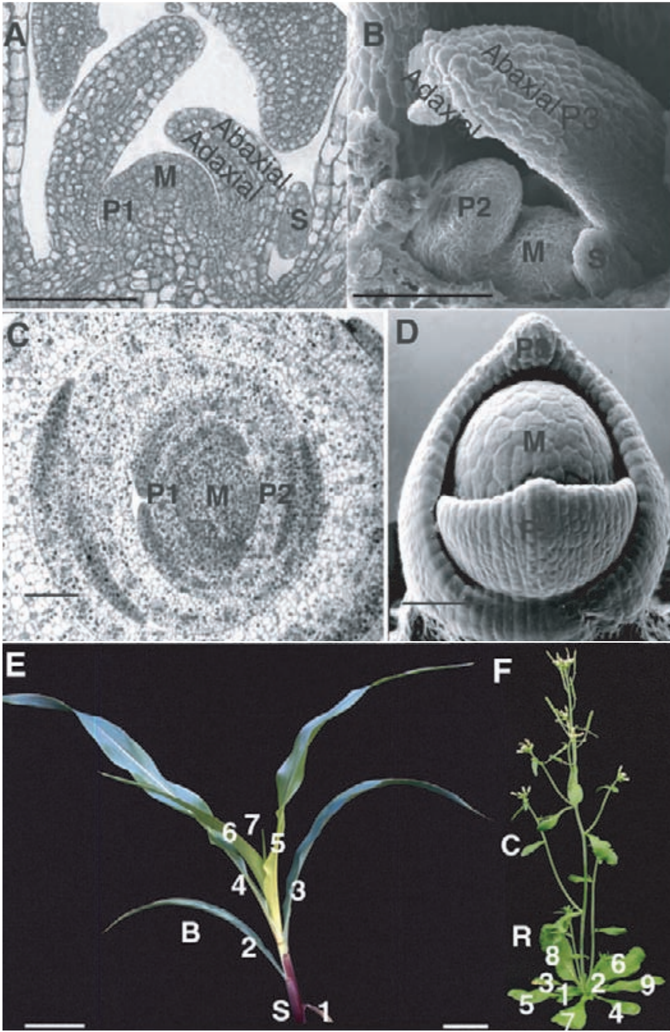


Plate 1. Leaf development in *Arabidopsis* and maize.

A. Longitudinal section through a five day-old *Arabidopsis* seedling. The bulge of the youngest leaf primordium (P1) can be seen on the left of the meristem dome (M). The adaxial and abaxial domains of the leaf are oriented relative to the SAM. A cross-section through a stipule (S) can be seen to the right of the meristem.

B. SEM of a seven-day-old *Arabidopsis* seedling. The leaf primordia curve over the meristem. Stipules (S) can be seen at the base of the plastochron 3 leaf (P3).

C. Transverse section through a two-week-old maize seedling. The leaves fully encircle the meristem. The margins of older leaves overlap.

D. SEM of a young maize meristem. Founder cells are recruited from around the meristem, so that the base of leaf primordia encircle the SAM. The *vorlauferspitze* is visible at the tip of the P2 and P3 leaves.

E. 14-day-old maize seedling. The alternate phyllotaxy is visible The leaves are numbered consecutively. The sheath (S) is red from anthocyanin; the leaf blades (B) are dark green with pale midribs.

F. Six-week-old *Arabidopsis* plant. The leaves are numbered consecutively. A rosette of vegetative leaves (R) with spiral phyllotaxy forms the base of the plant, the inflorescence stem is expanded and bears cauline leaves (C) and flowers.

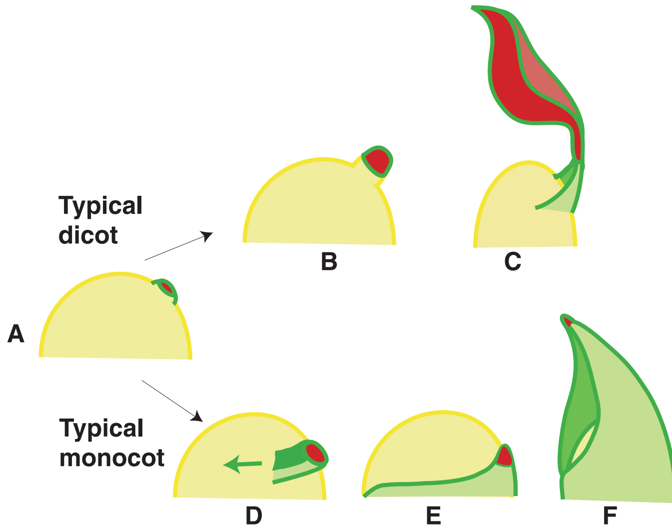


Plate 2. The contribution of the upper and lower leaf zones to the mature leaf in monocots and dicots.

At leaf initiation (A) the leaf primordium can be divided into a upper (red) and lower leaf zone. In most dicots the leaf grows out as a peg (B). Subsequently, dorsoventral patterning and mediolateral outgrowth occurs (C). The dicot leaf blade develops from the upper leaf zone, whereas the leaf base forms from the lower leaf zone (C). Leaf initiation in monocots starts on one side of the meristem (A). Dorsoventral patterning is established in the lower leaf zone before extensive outgrowth occurs (D). Cells around the perimeter of the meristem become recruited into the leaf primordium (E). The leaf sheath and blade of most dicots develops from the lower leaf zone. The upper leaf zone of the initial leaf primordium (red) rarely contributes to the growing leaf and can form a radial symmetric *vorlauferspitze* (F).

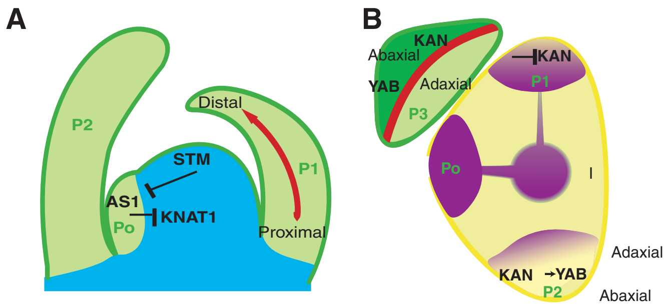


Plate 3. Genetic pathways regulating pattern formation during leaf development.

A. The *Arabidopsis knox* genes *STM1* and *KNAT1* are expressed in the meristematic cells of the SAM (blue). *STM* is expressed in the central zone of the meristem and in the internode regions. *KNAT1* is expressed in the peripheral zone of the meristem and in the internode regions. Both are down-regulated in during leaf initiation. *STM* is required to keep *AS1* out of the meristem. *AS1* is expressed in leaf primordia and is required to maintain down-regulation of *KNAT1* in the developing leaf.

B. *PHB* (purple) is expressed in the center of the meristem and in rays leading to the leaf primordia. At P0 *PHB* is expressed throughout the primordium (P0), but subsequently expression becomes gradually limited to the adaxial side. The *KANADI* and *YABBY* genes are also uniformly expressed initially, but expression becomes limited to the abaxial side in the P1 or P2 primordium. During P3, blade outgrowth begins at the boundary between *YABBY*-expressing domains and domains that do not express *YABBY* genes.

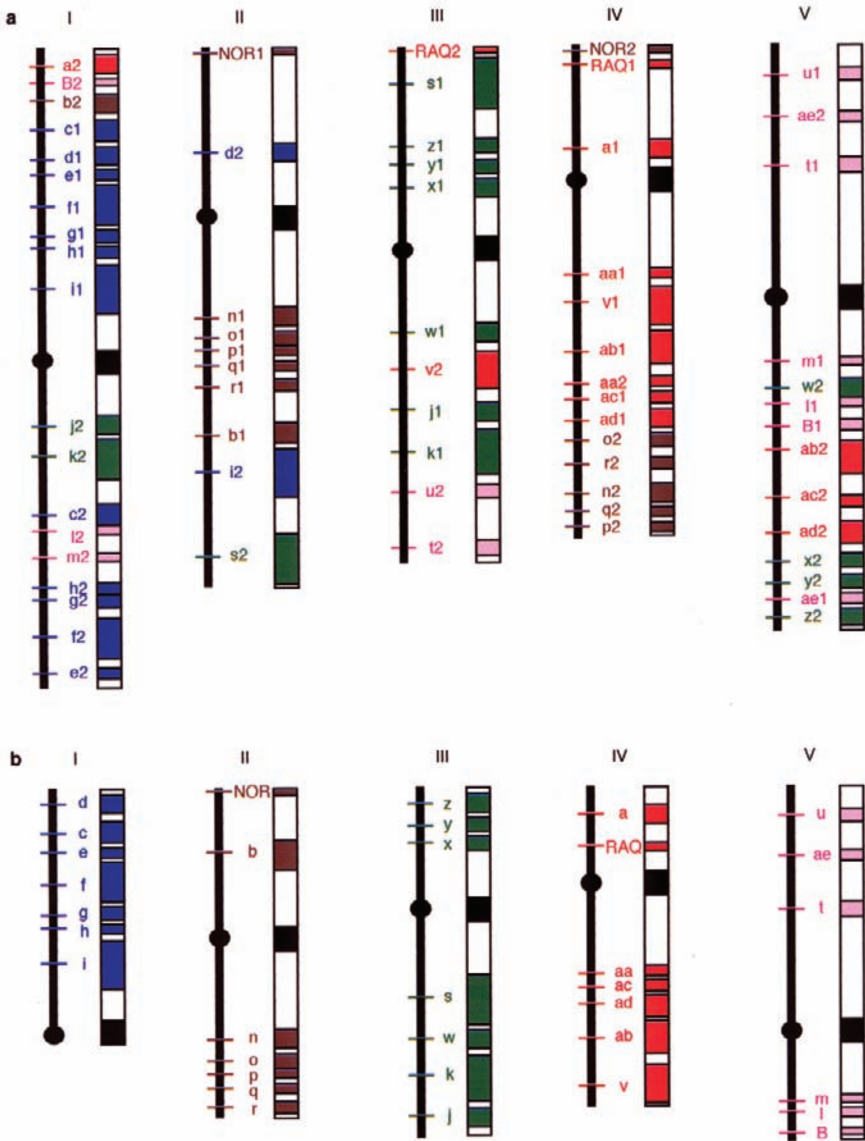


Plate 4. Schematic diagrams of the chromosomes and segments from present Arabidopsis thaliana (a) and from a putative Arabido-Brassica ancestor (b). For visual clarity, individual chromosomes are also depicted as vertical black bars, with their number at the top, centromeres are marked as black circles, and horizontal bars represent the various segments. Plate 4 (b) represents the haploid genome of a putative Arabido-Brassica ancestor prior to the events leading to the duplication of segments (Plate 4 (a)). The ancestral chromosomes possess a single copy of each segment. Thirty-four segments covering about 60% of the At genome were identified from data compiled from different sources (see the text). In order to maintain consistency with previous works, the submetacentric chromosome III is shown in an inverted position, with the short arm at the bottom and the long arm at the top.

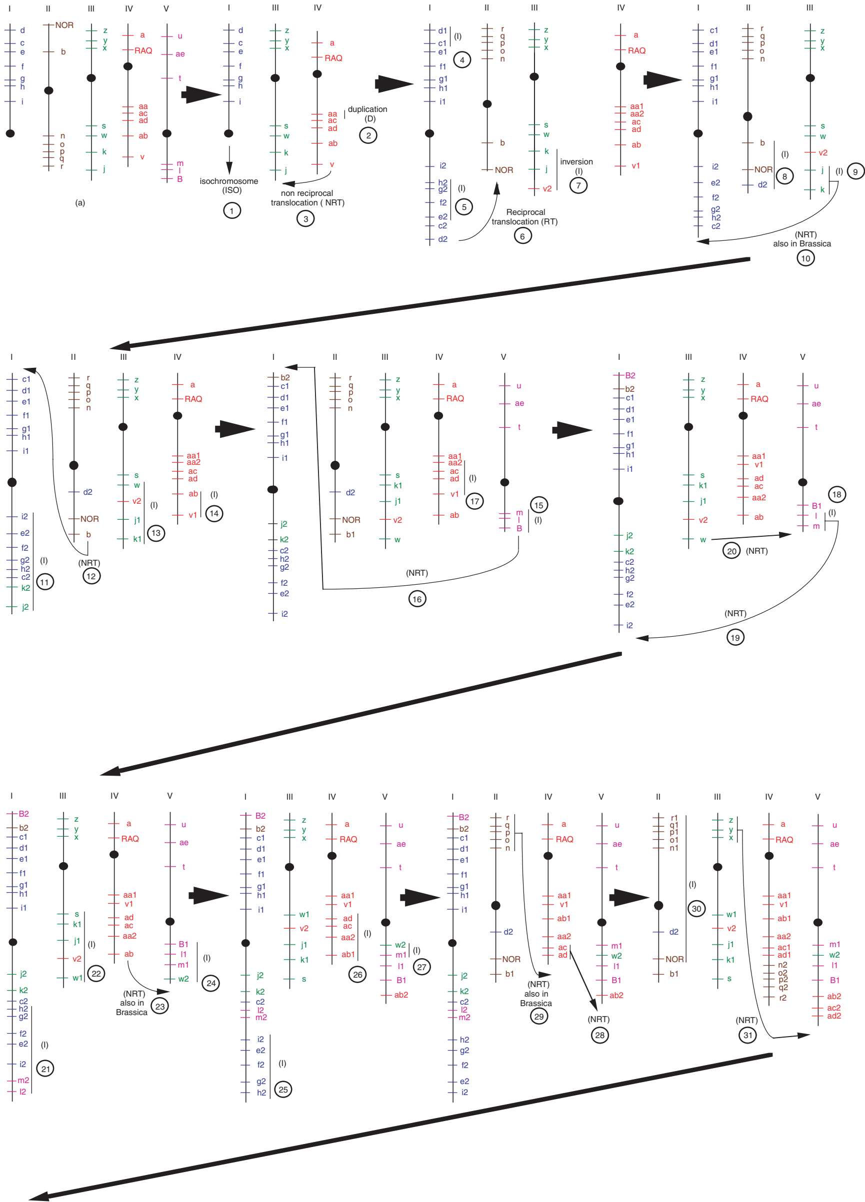


Plate 5. Successive duplications of segments and chromosomal rearrangements generating the present Arabidopsis thaliana genome organization (b) from a putative Arabido-Brassica ancestor (a). Plate 5 illustrates the evolutionary derivation of Arabidopsis chromosome segments and small blocks. In this model, 45 events (numbered 1 to 45) were introduced to account for successive duplications of segments and chromosomal rearrangements. ISO indicates isochromosome, NRT non-reciprocal translocation, D duplication, RT reciprocal translocation and I inversion.

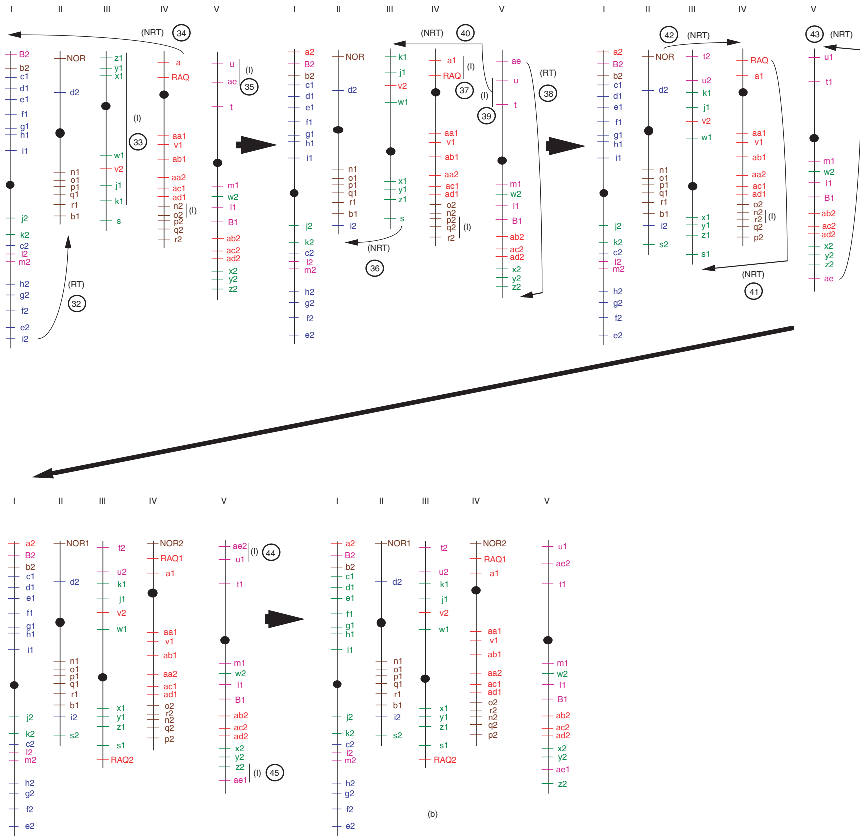


Plate 5 *(continued)*. We start from an ancestral genome (see Plate 4 (b)) consisting of five short chromosomes, with a telocentric chromosome I, a metacentric chromosome II, a submetacentric chromosome III and two acrocentric chromosomes IV and V. The series of rearrangements produces a metacentric chromosome I, two submetacentric chromosomes III and V and two acrocentric chromosomes II and IV. The short arm from the present chromosome V appears unchanged throughout the whole process. It is worth noting that the complex structure of chromosome I, and to a lesser extent chromosomes IV and V, requires more chromosomal rearrangements than, for example, the relatively simple structure of chromosome II.

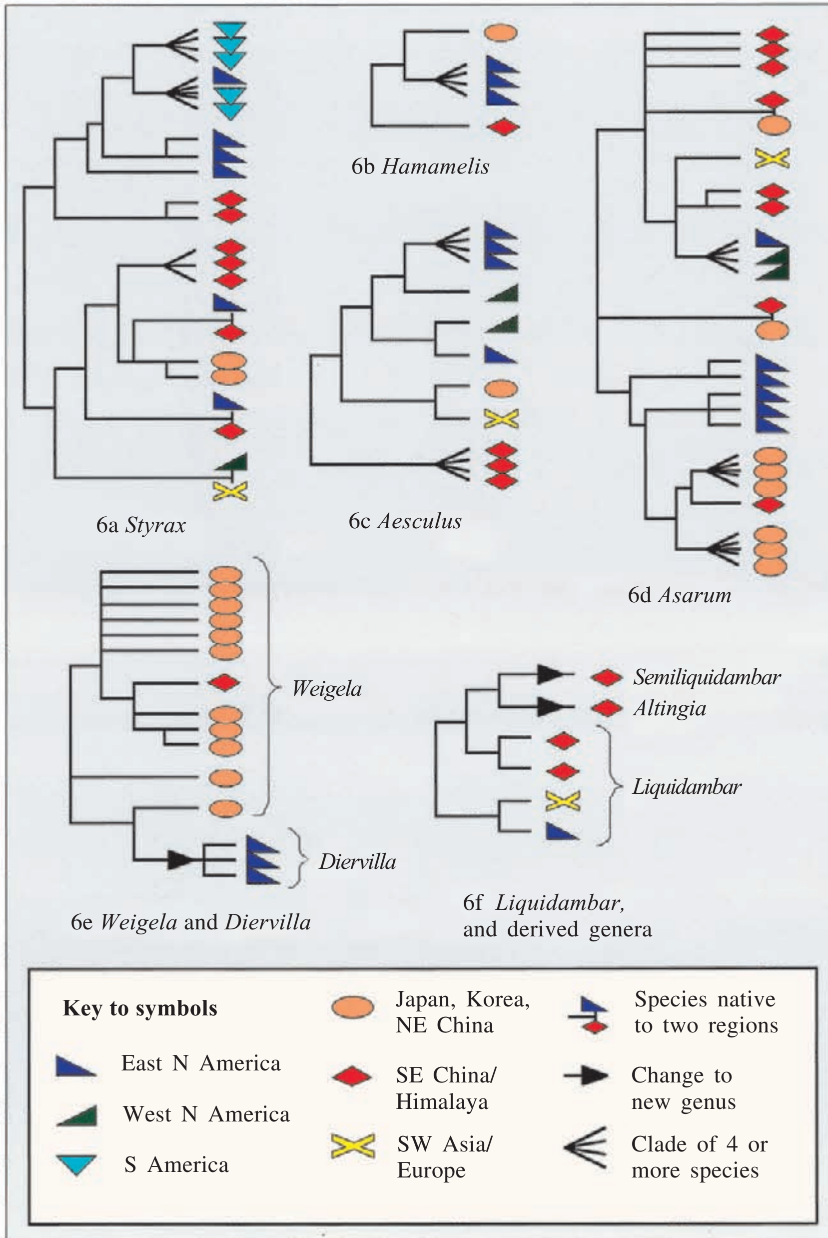


Plate 6. Area cladograms for groups exhibiting a Japan–China divide (**b**, **c** and **d**), a transatlantic sister relationship (**a** and **f**), and/or paraphyly (**e** and **f**). **6a** *Styrax* (Fritsch, 1999). **6b** *Hamamelis* (Wen and Shi, 1999). **6c** *Aesculus* (Xiang *et al.*, 1998a). **6d** *Asarum* (Kelly, 1998). **6e** *Weigela/ Diervilla* (Kim and Kim, 1999). **6f** *Liquidambar* (Shi *et al.*, 1998; Li and Donoghue, 1999). Symbols for area of occurrence replace species names, which in Plates 6a–e are in the same order as in the figures in each source reference.

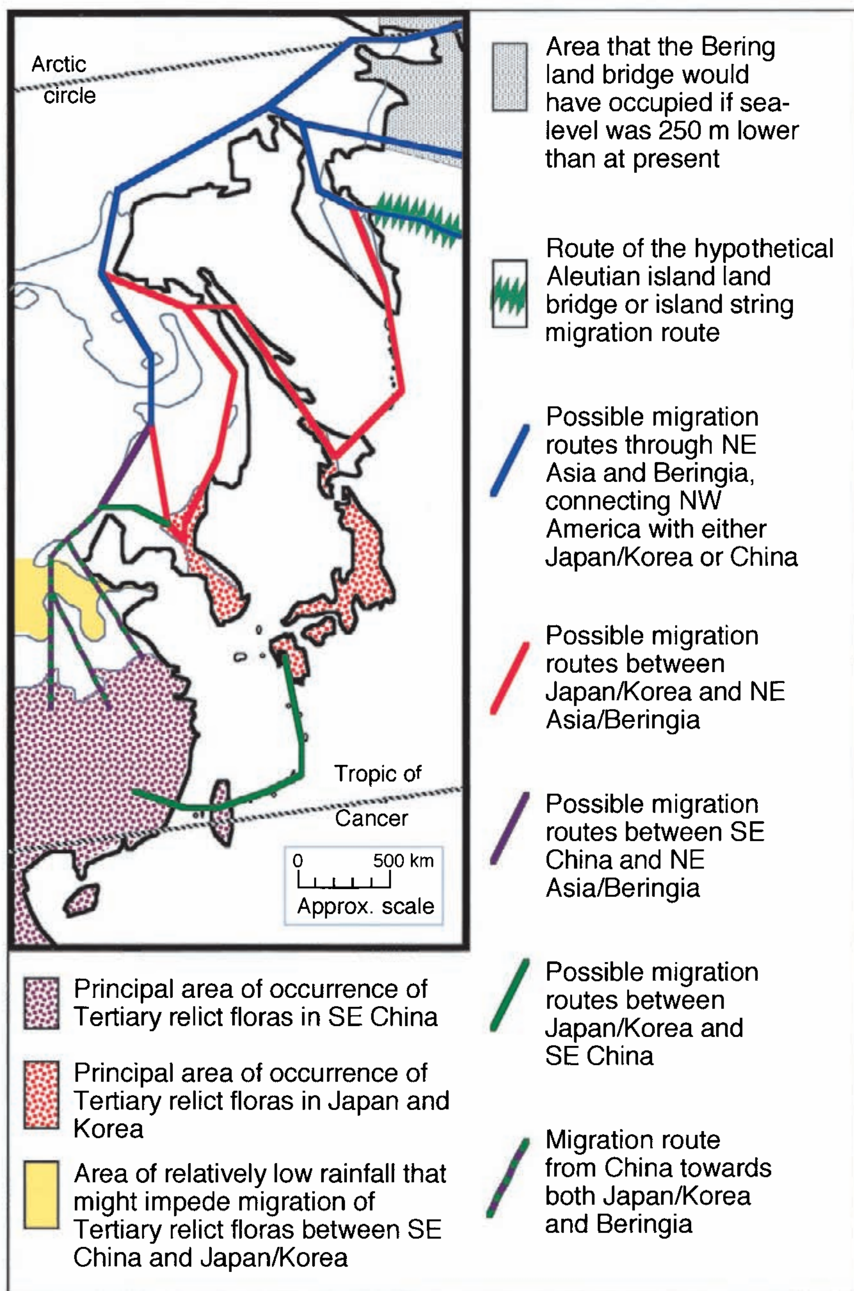
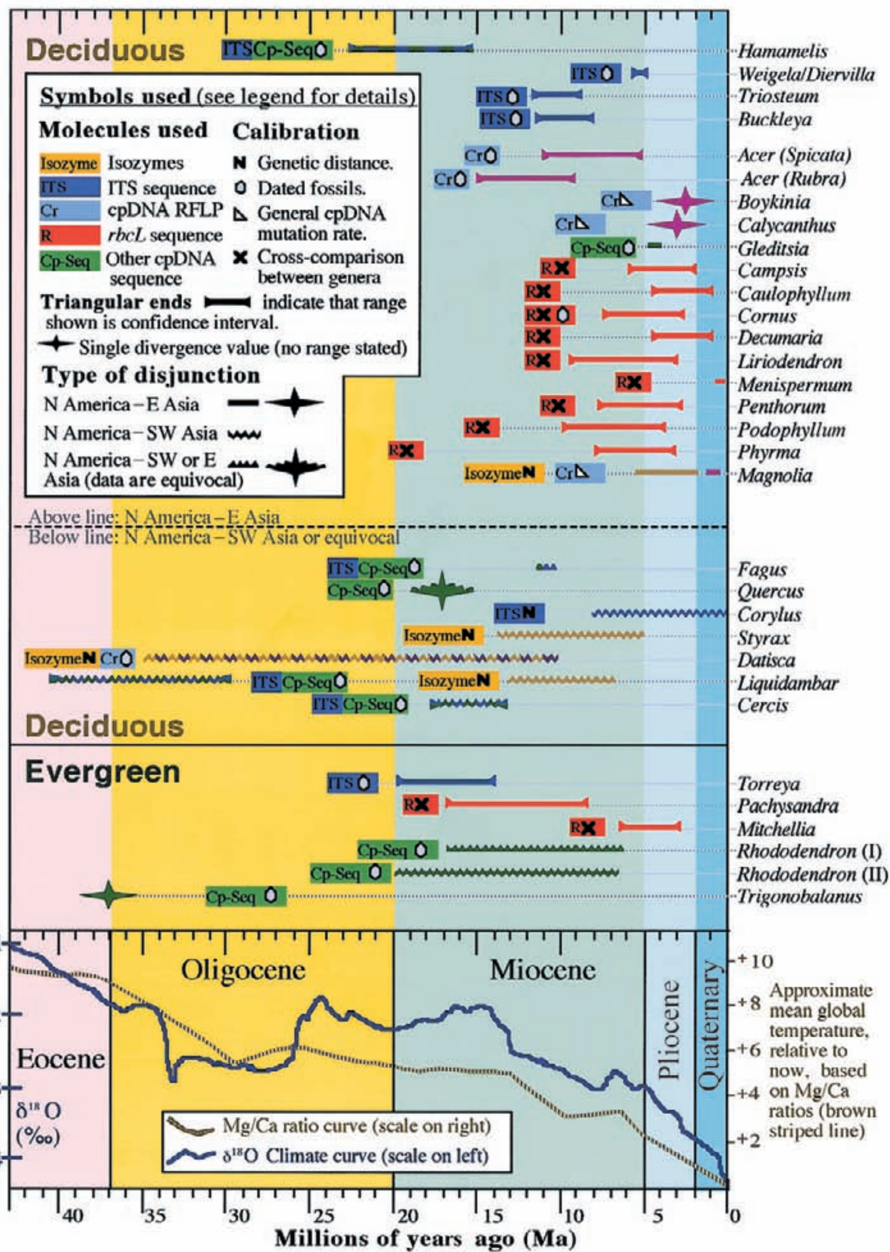


Plate 7. Possible migration routes that Tertiary relict floras employed between parts of East Asia and Beringia (and hence North America). Note that possible migration routes connecting SE China and Beringia all pass through or close to Japan/Korea, with no obvious phytogeographic barrier preventing a lineage undergoing such a migration from reaching Japan. Also shown is a second hypothetical migration route between Japan and China, via the Ryukyu Archipelago and Taiwan. Contours shown are for rainfall as in Fig. 2. Further inland (i.e. to the west) the annual rainfall is progressively reduced (Bartholomew, 1958).



Deciduous
Symbols used (see legend for details)
Molecules used
Isozyme Isozymes
ITS ITS sequence
Cr cpDNA RFLP
R rbcL sequence
Cp-Seq Other cpDNA sequence
Calibration
Genetic distance.
Dated fossils.
General cpDNA mutation rate.
Cross-comparison between genera
Triangular ends indicate that range shown is confidence interval.
Single divergence value (no range stated)
Type of disjunction
N America – E Asia
N America – SW Asia
N America – SW or E Asia (data are equivocal)
Hamamelis
Weigela/Diervilla
Triosteum
Buckleya
Acer (Spicata)
Acer (Rubra)
Boykinia
Calycanthus
Gleditsia
Campsis
Caulophyllum
Cornus
Decumaria
Liriodendron
Menispermum
Penthorum
Podophyllum
Phyrma
Magnolia
Above line: N America – E Asia
Below line: N America – SW Asia or equivocal
Fagus
Quercus
Corylus
Styrax
Datisca
Liquidambar
Cercis
Deciduous
Evergreen
Torreya
Pachysandra
Mitchellia
Rhododendron (I)
Rhododendron (II)
Trigonobalanus
Eocene
Oligocene
Miocene
Pliocene
Quaternary
δ18O (‰)
Mg/Ca ratio curve (scale on right)
δ18O Climate curve (scale on left)
Approximate mean global temperature, relative to now, based on Mg/Ca ratios (brown striped line)
Millions of years ago (Ma)

Plate 8. Divergence times of American–Eurasian disjuncts, and global climate deterioration during the last 43 million years.

Bars for each genus indicate divergence times between American species/clades and their closest Eurasian relatives. The ranges shown are published upper and lower limits of divergence times, and represent many different methods of range calculation; short bars do not necessarily represent more exact calculations. Ranges which are confidence intervals are marked with triangular ends. Colours of bars indicate molecules used in making an estimate (see symbols box). Two-coloured bars indicate divergence estimates derived from two molecules combined. For *Magnolia* and *Liquidambar*, two divergence estimates based on different molecules are shown. Type of bar (straight, zigzag or toothed) indicates type of disjunction as shown in box. Other cpDNA sequences employed were as follows: *MatK* for *Rhododendron* I and II; *ndhF* and *rpl*16 for *Gleditsea*; *ndhF* for *Cercis trnL-F*, *MatK* and GBSS (waxy) for *Hamamelis* and *Liquidambar*; *rbcL/atpBE* intergenic spacer and 5′ *trnK* intron for *Fagus; rbcL/atpBE* intergenic spacer and 3′*trnK* intron for *Quercus*; 5′*trnK* intron and 3′*trnK* intron for *Trigonobalanus*.

Calibration by genetic distance used the equations of Nei (1972) and/or Sarich (1977) to estimate time since divergence. 'Dated fossils' indicates that a fossil of known age corresponding with a branch in the phylogeny or the origin of an extant group was used to calibrate the rate of molecular substitution in the genus examined. 'General cpDNA mutation rate' indicates that a general rate calculated for woody angiosperms was employed to estimate times since divergence. Cross-comparisons between genera in all cases used a molecular clock constructed for *Cornus* to calibrate substitution rates, plus relative rate tests and rate corrections, and estimated divergence times using synonymous substitutions only Xiang *et al.* (2000).

Sources of data are as follows. *Acer* – Hasebe *et al.* (1998); *Hamamelis, Weigela/Diervilla, Triosteum, Buckleya, Liquidambar* (ITS and cpDNA data), *Cercis* and *Torreya* – Donoghue *et al.* (2001); *Boykinia* and *Calycanthus* – Xiang *et al.* (1998b); *Gleditsea* – Schnabel and Wendel (1998); *Campsis, Caulophyllum, Cornus, Decumaria, Liriodendron, Menispermum, Penthorum, Podophyllum, Phyrma, Pachysandra* and *Mitchellia* – Xiang *et al.* (2000); *Magnolia* (section *Rytidospermum*; both estimates) – Qiu *et al.* (1995b), note cpDNA divergence range halved from original paper according to note in Xiang *et al.* (2000); *Quercus* (section *Quercus*), *Fagus* and *Trigonobalanus* – Manos and Stanford (2001); *Corylus* (section *Corylus*) – Whitcher and Wen (2001); *Styrax* – Fritsch (1996); *Datisca* – see Table I; *Liquidambar* (isozyme) – Hoey and Parks (1991); *Rhododendron* – R. I. Milne, unpublished data. *Rhododendron* (I) indicates divergence of *R. catawbiense* and *R. macrophyllum* (America) from *R. caucasicum* (SW Asia), *R. brachycarpum* and *R. aureum* (East Asia). *Rhododendron* (II) indicates divergence of *R. maximum* (America) from *R. ponticum* and *R. ungernii* (SW Asia).

Palaeoclimate curves constructed from two methods are shown. $\delta^{18}O$ is a widely used indicator of palaeoclimate, and large sample sizes mean that sampling error is minimal (Zachos *et al.*, 2001). However, $\delta^{18}O$ is affected by both temperature and ice volume, and is thus not an absolute indicator of global temperature (Zachos *et al.*, 2001). Conversely, Mg/Ca ratios record temperature changes alone, but this is a new technique, so sample sizes as yet are small, creating a greater potential for sampling error (Lear *et al.*, 2000).